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(54) Title: MUTANTS OF THE Rb AND p53 GENES AND USES THEREOF

(57) Abstract

The present invention provides mutants of the Rb and p53 genes and methods utilizing these mutants therapeutically. Along with mutated Rb genes and p53 genes, the present invention provides plasmids containing a mutated Rb gene or a p53 gene. In addition, the present invention provides cells transfected with the plasmids of the present invention. Moreover, the present invention provides for methods of treating a variety of pathophysiological cell proliferative diseases.

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MUTANTS OF THE Rb AND p53 GENES AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

5   The present invention relates generally to the field of regulation of cell growth and proliferative diseases. More specifically, the present invention relates to mutants of the RB-1 and p53 genes and therapeutic uses of such mutants.

Description of the Related Art

10   The control of cell proliferation is a complex process which involves multiple interacting components. Whether a cell grows or not depends on the balance of the expression of negatively-acting and positively-acting growth regulatory genes. Negatively-acting growth regulatory genes are those that, when

15   expressed in or provided to a cell, lead to suppression of cell growth. Positively-acting growth regulatory genes are those which, when expressed in or provided to a cell, stimulate its proliferation.

Recently, several negatively acting growth regulatory

20   genes called tumor suppressor genes which have a negative effect on cell proliferation have been identified. These genes include, but are not limited to, the human retinoblastoma gene, RB-1, and the p53 gene. The absence or inactivation of some of these negative growth regulatory genes has been correlated with certain

25   types of cancer.

The human retinoblastoma gene, RB-1, is the prototype of this class of tumor suppressor genes in which the absence of both alleles of the gene in a cell or the inhibition of the expression of the gene or its gene product will lead to

30   neoplastic or abnormal cellular proliferation. At the molecular level, loss or inactivation of both alleles of RB-1 is involved in the clinical manifestation of tumors such as retinoblastoma and clinically related tumors, such as osteosarcomas, fibrosarcomas, soft tissue sarcomas and melanomas. In addition,

35   loss of the function of RB-1 has also been associated with other

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types of primary cancer such as primary small cell lung carcinoma, bladder carcinoma, breast carcinomas, cervical carcinomas and prostate carcinomas.

The Tumor Suppressor Genes Rb and p53 suppress the proliferation of cancerous and noncancerous cells

5   The re-introduction of a wild-type cDNA of RB-1 or p53 have been shown to partially restore normal growth regulation as the re-introduced genes induce growth arrest or retardation in many different tumor cell types. The designation of the Rb gene as a tumor suppression gene stemmed from the fact that 10   inactivation of an allele of the Rb gene is a predisposition to the development of cancer. However, the growth suppression effect of the Rb gene is not restricted to tumor cells. Normal cells which have two copies can be growth arrested or retarded by the introduction of extra copies of the Rb gene under certain growth 15   conditions. Likewise, the ability of a wild type p53 to suppress the growth of noncancerous cells is well documented. Thus, the step controlled by Rb and p53 may not directly affect the tumorigenic phenotype but rather the steps that control the 20   growth of tumor and normal cells alike are affected.

Mechanisms of pathological proliferation of normal cells

There is a wide variety of pathological cell proliferative conditions for which novel methods are needed to provide therapeutic benefits. These pathological conditions may 25   occur in almost all cell types capable of abnormal cell proliferation. Among the cell types which exhibit pathological or abnormal growth are (1) fibroblasts, (2) vascular endothelial cells, and (3) epithelial cells. It can be seen from the above that methods are needed to treat local or disseminated 30   pathological conditions in all or almost all organ and tissue systems of the individual.

For instance, in the eye alone, novel methods may be utilized to treat such a wide variety of pathologic disease states which are due to abnormal proliferation of normal, benign 35   or malignant cells or tissues including, but not limited to, the following: fibroproliferative, vasoproliferative and/or neoplastic diseases of the eye: retinopathy of prematurity,

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proliferative vitreoretinopathy, proliferative diabetic retinopathy, capillary hemangioma, choroidal neovascular nets, subretinal neovascular nets, senile macular degeneration due to subretinal, neovascularization, corneal neovascularization,

5   macular pucker due to epiretinal membrane proliferation, adult cataracts due to nuclear sclerosis, fibrous ingrowth following trauma or surgery, optic nerve gliomas, angiomatosis retinae, neovascular glaucoma, cavernous hemangioma, rubeosis iridis, sickle cell proliferative retinopathy, epithelial downgrowth

10 after eye surgery or injury, after-cataract membrane, papilloma, retinal neovascularization in thalassemia, subretinal neovascularization due to pseudoxanthoma elasticum, and neurofibromatosis type 1 and 11, retinoblastoma, uveal melanoma, and pseudotumor of the orbit. Other benign cell proliferative

15 diseases for which the present invention is useful include, but are not limited to, psoriasis, ichthyosis, papillomas, basal cell carcinomas, squamous cell carcinoma, and Stevens-Johnson Syndrome.

      It should be noted that in the case of normal cells,

20 there are already two normal alleles each of the Rb gene and the P53 gene and yet these wild type proteins fail to prevent the cells from proliferating when the cells were provided growth factors, as for example in tissue culture growth conditions. The uncontrolled proliferation of otherwise resting cells in

25 pathological conditions is also due to the exposure of the cells to growth factors induced by the pathological conditions (see below). For an example, uncontrolled proliferation of blood vessels in the eye in thalassemia can lead to detachment of the retina if the proliferation is not stopped. The introduction of

30 extra copies of the Rb gene or the P53 gene into such cells may or may not be sufficient to suppress the cells from growing. In fact, the introduction of exogenous Rb gene into many tumor cells only retarded the growth rate of the cells instead of complete arrest. The previously reported cell lines such as Saos-2 and

35 DU145 are good examples of this phenomenon. Many more copies of the gene may need to be introduced and it is difficult to control on the one hand the number of copies that need to be or could be

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introduced. On the other hand, growth factor exposure may lead
to inactivation of the expressed Rb proteins.

Regulation of the Activity of Rb Protein by Phosphorylation

If the inactivation of growth suppressor genes is an
essential step in the removal of the constraint on cell growth,
there must be mechanisms whereby the activities of these gene
products are regulated so that a cell may proliferate in a
controlled manner. It is conceivable that the expression of a
given growth suppressor gene may be regulated quantitatively or
qualitatively. In the case of the Rb gene, the ratio of the
steady state level of the protein to the cell volume is a
constant throughout the cell cycle. This lack of variation of
the Rb protein concentration suggests that there must be other
mechanisms whereby a cell can regulate the activity of this
protein. There are several lines of evidence to show that the
activity of the Rb protein (pRb) is regulated by its
phosphorylation state. Various growth stimulatory or inhibitory
factors exert their effects by perturbing the phosphorylation of
growth suppressor gene products such as the Rb protein. Evidence
for a role of growth stimulatory factors in the induction of
phosphorylation of the Rb proteins have come from the observation
that the Rb proteins exist in the underphosphorylated forms in
quiescent cells. In addition, when quiescent cells were
stimulated to proliferate by exposure either to serum or to
growth factors such as EGF together with insulin and
transferrin, the predominant form of the Rb protein was
underphosphorylated in G1 but became hyperphosphorylated as the
cells enter the G1/S boundary and the cells. These data suggest
that the Rb protein is a target of the signal transduction
pathway induced by serum or growth factors. Finally, senescent
human fibroblast cells incapable of responding to the
proliferation stimulatory effects of growth factors also failed
to phosphorylate the Rb protein.

There are also evidence for a role of growth inhibitory
factors in the downregulation of phosphorylation of the Rb
proteins. When actively growing leukemia or neuroblastoma cells
were treated with retinoic acid or vitamin D3, DMSO other

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differentiation inducing reagents, the Rb proteins were found to be underphosphorylated prior to the on set of cell growth arrest in the early G1 phase and induced to differentiation.   In addition, the treatment of lung epithelial cells with the
5   paracrine growth inhibitory polypeptides-transforming growth factor Beta 1 (TGFβ-1) led to the downregulation of phosphorylation of the Rb protein and concomitant arrest of cell growth in late G1.   Taken together, these data suggest the underphosphorylated form of the Rb protein actively suppressed
10   cells from traversing the G1/S boundary and that a kinase (S), activated in G1, can inactivate the Rb protein so that the cells may move into the S phase.

          While the permanent removal of the Rb gene, by deletion or mutation, in tumor cells allowed growth, removal of the
15   constraint on growth in normal cells having normal expression of the Rb gene is achieved by cell cycle dependent post-translational modification of its gene product, i.e., the Rb protein (pRb).   The Rb protein exists in multi-phosphorylated forms and evidence suggests that the underphosphorylated form is
20   the active form.   For example, the SV40 large T antigen preferentially binds to the underphosphorylated form of the Rb protein. Phosphorylation of the Rb protein releases it from the SV40 large T antigen and only the underphosphorylated forms of pRb binds to and inhibits transcription from the E2 promotor.
25   Thus, the underphosphorylated form of the Rb protein actively suppresses cell growth, whereas cell proliferation is associated with hyperphosphorylation of the Rb protein.   Various growth stimulatory or inhibitory factors may exert their effects by perturbing the phosphorylation of the Rb protein.   Therefore,
30   although a normal cell may be expressing the Rb proteins, it can be induced to grow when exposed to appropriate growth factors such as may be found in various growth conditions, including pathological conditions. Under certain pathological conditions, an otherwise normal cell may be induced to proliferate
35   undesirably. Proliferation of normally quiescent fibroblasts in the eyes in diabetic retinopathy is an example of such unwanted induction of cell growth.   Left untreated, the proliferating

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fibroblasts will eventually attach to the retina and lead to its
rapid detachment.

Activation of the p53 protein

          Similar to the Rb gene, inactivation or mutation of the
5   p53 gene has been frequently observed in tumor and nontumorous
cell lines. Wild type p53 protein is also negatively regulated
when it binds to viral antigens such as the SV40 large T. The
p53 protein functions as a transcription factor and binds to
specific DNA sequences. The ability of the wildtype p53 protein
10   to bind DNA is critical for it to function properly. In mutant
p53, the ability of the protein to bind to specific DNA target
sequences or to activate transcription is lost, suggesting that
these activities are important for the suppressive function of
the protein. However, it has also been recently shown that
15   sequence specific DNA binding activity of the p53 protein is
cryptic. Newly synthesized p53 protein is inactive in that it
cannot bind to its specific DNA sequence unless the protein is
modified. One of the sites for modification is amino acid
residues 365-393 at the C-terminus. This region is the site for
20   the attachment of an RNA moiety as well as for the binding to the
bacterial heat shock proteins dnaK. Binding of an antibody,
Pab421, to the 365-389 amino acid residues activates the protein
by altering its conformation so that it can bind to its specific
DNA sequence.

Difference in the mode of function of pRb and p53

25   Although both Rb and p53 are regarded as tumor
suppressor genes, their modes of action are very different.
Previously, evidence was provided that these two genes may work
on different pathways.   Mice null for the Rb gene is
30   embryonically lethal while mice null for p53 develop normally
although they are more susceptible to cancer development. At the
cellular level, cancer cells that are devoid of p53 can be growth
arrested or retarded by the overexpression of the Rb gene even
in the absence of exogenous p53. On the other hand, cells devoid
35   of Rb are susceptible to the growth suppression effect of the p53
gene.   At the molecular level, p53 and pRb interacts with

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different protein targets and DNA sequences (directly or indirectly).

The use of the Rb and p53 gene in therapy

5   While these two proteins are different in their modes of action, they are similar in one aspect, i.e., overexpression of the active forms of the two proteins under experimental conditions lead to the suppression or retardation of growth of cells. Knowing that cell growth is controlled by the expression of suppressor genes such as pRb and p53 and that their proteins 10   need to be in the active conformations in order to function, it is desirable to have active forms of these genes for gene therapy. In the case of pRb, the newly synthesized proteins are underphosphorylated and are active. Inactivation may occur as the cells are exposed to growth factors. It would be desirable to 15   have mutant forms of pRb that would remain in the active form and are resistant to inactivation by phosphorylation. In the case of p53, the newly synthesized protein is inactive in that it cannot bind DNA unless modified. In gene therapy, it would be highly desirable to have mutant forms of the p53 gene, the 20   protein of which is already active even without further modification. Finally, since the two proteins have different modes of action, it would be desirable to use a combination of both the Rb and the p53 genes in therapy such that the mutations or growth conditions that renders a cell resistant to the growth 25   suppression effect of one gene may be susceptible to the other.

The prior art remains deficient in the lack of a tumor suppressor gene mutated so that the gene product (protein) is permanently active and can function even when the cell is exposed to growth factors. More specifically, the prior art is deficient 30   in the lack of effective and functional mutated forms of the p53 and Rb genes. The prior art also remains deficient in the simultaneous application of the wildtype and/or the mutant forms of the Rb and the p53 genes in cancerous and noncancerous cell proliferative diseases.

35   SUMMARY OF THE INVENTION

In one aspect, the present invention provides a generalized approach to the treatment of inappropriate or

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pathological cell growth such as exists in non-cancerous cell
proliferative diseases as well as cancerous cell proliferative
diseases.   More specifically, the present invention provides
methods of treating pathophysiological cell proliferative
5   diseases by administering mutated growth suppressor gene and /or
gene products, i.e., mutated proteins.

In one embodiment of the present invention, there is
provided an isolated DNA molecule, wherein said molecule is a
synthetic mutated Rb gene encoding a mutated functionally active
10 retinoblastoma protein.

In another embodiment of the present invention, there
is provided an isolated DNA molecule, wherein said molecule is
a synthetic mutated p53 gene encoding a mutated functionally
active p53 protein.

15 In another embodiment of the present invention, there
is provided a novel plasmid containing a functionally active
mutant of the retinoblastoma or p53 gene.

In yet another embodiment of the present invention,
there is provided a method of the consecutive or simultaneous
20 administration of the wild type and/or the active mutants of both
the Rb and the p53 genes for therapeutic purposes.

In other embodiments of the present invention, there
are provided methods for the treatment of pathological cell
proliferative diseases comprising administration concurrently or
25 consecutively to a non-cancerous proliferating cell a mutated Rb
gene and a mutated P53 gene.

Additionally provided is a method of treating malignant
cell   diseases   in   individuals   comprising   administration
concurrently or consecutively into a proliferating cancer cell
30 of a mutated Rb gene and a mutated P53 gene.

In yet another embodiment of the present invention,
there is provided a method of inducing apoptosis, comprising the
step of administering the mutant p53 protein of the present
invention to a cell.

35 In yet another embodiment of the present invention,
there is provided a method of treating a tumor cell having a

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mutation of a cyclin protein, comprising the step of administering the protein of claim 39 to a cell.

Other and further aspects, features, and advantages of the present invention will be apparent from the following
5   description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the discovery of the homology region between pRb and p53 by immunoprecipitation of cellular proteins
10   using polyclonal anti-Rb antibodies RB1-Ab #18 and #20.

Figure 2 shows the confirmation of the homology region between pRb and p53 immunoprecipitation of cellular protein using polyclonal anti-Rb antibodies RB1-Ab #18 and #20.

Figure 3 shows the ability of the P1, P3, P5 mutant p53
15   proteins to bind specific DNA sequences in the absence of Pab421.

Figure 4 shows the two dimensional mapping analysis of the phosphorylation pattern of mutant Rb proteins as compared to those of the wildtype, (A) in vivo labelled proteins (B) in vitro labelled proteins.

20   Figure 5 shows the binding of the retinoblastoma protein to the SV40 Large T antigen and the dissociation of the complex by phosphorylation.

Figure 6 shows the two dimensional mapping analysis of two different forms of phosphorylated retinoblastoma protein
25   (those that are bound by SV40 large T and those that are not).

Figure 7 shows the two dimensional mapping analysis of the change in the phosphorylation pattern of the retinoblastoma protein under different growth conditions.

Figure 8 shows the ability of the m89 Rb mutant to
30   suppress the growth of the normal cell WS1.

Figure 9 shows the ability of the m89 Rb mutant to suppress the growth of the bladder tumor cell line TCCSUP.

Figure 10 shows a schematic diagram of both the wild type p53 protein showing the domain F and domain R and the mutant
35   p53 protein showing the domain F and domain R.

Figure 11 shows that the mutant p53 protein is able to bind specific DNA sequences.

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Figure 12 shows that the homology regions control the exposure of the C-terminus of p53 so that specific sites are not phosphorylated.

Figure 13 shows that the homology region mutants of p53 are transcriptionally active *in vivo*. Figure 13A shows that p53 mutants of the p3 or p5 homology region at the conserved aminoacids is active in promoting transcriptional activation. Figure 13B demonstrates that equal amount of proteins were used for all the p53 constructs.

Figure 14 shows that the synthetic p53 mutant induces apoptosis in the lung carcinoma cell line, H358. Figure 14A, wild type p53 without cisplatin; Figure 14B, wild type p53 + cisplatin; Figure 14C, P4 p53 mutant without cisplatin; Figure 14D, P4 p53 mutant + cisplatin; Figure 14E, P5 p53 mutant without cisplatin; Figure 14F, P5 p53 mutant + cisplatin.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "functional expression of the gene" is meant to include the suppression of transcription of the gene, the degradation of the gene transcript (pre-message RNA), the inhibition of splicing, the destruction of the message RNA, the prevention of translation of the message RNA, the prevention of the post-translational modifications of the protein, the destruction of the protein, or the inhibition of the normal function of the protein.

The term "transfected plasmid" is meant to include the bacterial plasmid which contains a mutated retinoblastoma or p53 gene to be carried (transfected) into the cell of choice.

The term "gene therapy" is meant to include the insertion of part or all of a gene, a DNA construct, RNA, or gene product into a cell, group of cells, tissue, pathologic lesion, organ or organism for the purpose of modulating gene expression, and/or function of the gene product.

The term "prophylactic gene therapy" is meant to include genes which may be used for partial or total inhibition or prevention of disease and the spread of disease and also is meant to include genes which may be used to supplement or replace

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absent or defective negative growth in cell, tissues or germlines.

The term "cell proliferative disease" is meant to include any human or animal disease or disorder, affecting any one or any combination of organs, cavities or body parts, which is characterized by single or multiple local abnormal proliferations of cells, groups of cells or tissue(s), whether benign or malignant.

The term "prokaryote" is meant to include all bacteria which can be transformed with the DNA for the expression of the recombinant molecules of the present invention.

The term "eukaryote" is meant to include all yeasts, fungi, animal and plant cells which can be transformed with the DNA for the expression of the recombinant molecules of the present invention.

The DNA for DNA constructs of the present invention can be synthetic or may be derived from any mammalian species. All that is required is that the genetic sequence for the Rb or p53 genes be functionally expressed in the prokaryotic or eukaryotic organism. Preferred is synthetic DNA.

A recombinant DNA molecule coding for the DNA constructs of the present invention can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art.

Methods for preparing fused, operably linked genes and expressing them in bacteria are known and are shown, for example, in U.S. Patent No. 4,366,246, herein incorporated by reference. The genetic constructs and methods described therein can be utilized for construction of the DNA constructs of the present invention and transfection in prokaryotic or eukaryotic hosts.

Prokaryotic hosts may include Gram negative as well as Gram positive bacteria, such as *E. coli*, *S. tymphimurium*, *Serratia marcescens* and *Bacillus subtilis*. Eukaryotic hosts may include yeasts such as *Pichia pastoris* or mammalian cells.

In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted DNA fragment are used in connection with the host. The

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expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to
5   means known in the art to achieve optimal cell growth.

Examples of promoters which can be used in the invention include, but are not limited to: human β-actin promotor, metallothionin promotor, SV40 origin of replication, MMTV LTR promotor and MuLV LTR promotor. Examples of some of the
10   plasmids or bacteriophage which can be used in the invention are listed in Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratories, 1982, and others are known to those of skill in the art and can be easily ascertained.

A gene is a DNA sequence which encodes through its
15   template or messenger RNA a sequence of amino acids characteristic of a specific peptide. The term cDNA includes genes from which the intervening sequences have been removed. The term "recombinant DNA" (rDNA) is meant to include a molecule that has been recombined by splicing cDNA or genomic DNA
20   sequences in vitro.

A cloning vehicle is a plasmid or phage DNA or other DNA sequence which is able to replicate in a host cell which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a
25   determinable fashion without loss of an essential biological function of the DNA, and which contains a marker suitable for use in the identification of transformed cells. Markers, for example, are tetracycline resistance or ampicillin resistance. The word "vector" is sometimes used for a cloning vehicle.

30   An expression vehicle is a vehicle similar to a cloning vehicle but which is capable of expressing a given structural gene in a host, normally under control of certain control sequences.

The term "individual" is meant to include animals and
35   humans.

The term "biologically inhibiting" or "inhibition" of the growth of proliferating cells is meant to include partial or

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total growth inhibition and also is meant to include decreases in the rate of proliferation or growth of the cells. The biologically inhibitory dose of the mutants of the present invention may be determined by assessing the effects of the test
5   element on target malignant or abnormally proliferating cell growth in tissue culture, tumor growth in animals and cell culture or any other method known to those of ordinary skill in the art.

As used herein, the term "conserved homology region"
10   refers to that amino acid region that is homologous between Rb and p53 as illustrated in Table I.

Administration of the proteins of the present invention may be by topical, intraocular, parenteral, oral, intranasal, intravenous, intramuscular, subcutaneous, or any other suitable
15   means. The preferred method of administration for treatment of ocular diseases is intraocular or periocular injection. The preferred method of administration for treatment of skin cell proliferative diseases is by topical application or subcutaneous injection.

20   In another embodiment, the DNA constructs of the present invention may be delivered to the focal proliferative disease directly. In the case of ocular proliferative disease, the DNA constructs of the present invention may be directly injected into the eye. The DNA constructs of the present
25   invention may be delivered directly to focal disease sites in internal organs, body cavities and the like by use of imaging devices used to guide the injecting needle directly to the disease site. The DNA constructs of the present invention may also be administered to disease sites at the time of surgical
30   intervention.

The DNA dosage administered is dependent upon the age, clinical stage and extent of the disease or genetic predisposition of the individual, location, weight, kind of concurrent treatment, if any, and nature of the pathological or
35   malignant condition. The effective delivery system useful in the method of the present invention may be employed in such forms as capsules, tablets, liquid solutions, suspensions, or elixirs, for

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oral administration, or sterile liquid forms such as solutions,
suspensions or emulsions. Any inert carrier is preferably used,
such as saline, or phosphate-buffered saline, or any such carrier
in which the compounds used in the method of the present
5   invention have suitable solubility properties.

Preferably, for intraocular delivery and for delivery
to other localized disease sites, delivery systems useful in the
method of the present invention may be employed in such sterile
liquid forms such as solutions, suspensions or emulsions. For
10   topical use it may be employed in such forms as ointments, creams
or sprays. Any inert carrier is preferably used, such as saline,
or phosphate-buffered saline, or any such carrier in which the
compounds used in the method of the present invention have
suitable solubility properties.

15   For local administration to cells, administration may
be by any method known to those of skill in the art including,
but not limited to, transfection, electroporation, microinjection
of cells, or in vehicles such as liposomes, lipofectin or as
naked DNA or RNA. The DNA of the present invention may be
20   delivered by known gene delivery systems such as, but not limited
to, retroviral vectors (Gilboa (1982) J. Virology 44:845; Hocke
(1986) Nature 320:275; Wilson et al., Proc Natl Acad Sci USA
85:3014), vaccinia virus system (Chakrabarty et al., (1985) Mol.
Cell Biol. 5:3403) or other efficient DNA delivery systems (Yates
25   et al., (1985) Nature 313:812) known to those of skill in the
art. These references are exemplary only. In order to
specifically deliver or transfect cells which are abnormally
proliferating and spare non-dividing cells, it is preferable to
utilize a retrovirus delivery system known to those of skill in
30   the art. Since host DNA replication is required for retroviral
DNA to integrate and the retrovirus will be unable to self
replicate due to lack of the retrovirus genes needed for its life
cycle, utilizing such a retroviral delivery system the present
invention will target said anti-proliferative elements to
35   abnormally proliferating cells and will spare non-dividing normal
cells from any intervention.

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The mutant proteins of the present invention may be administered in any biologically effective carrier. The biologically effective carriers may include retroviruses, liposomes, and any other transfection mechanism capable of 5 introducing foreign proteins into the genome of the cell. Such transfection mechanisms are known to those of skill in the art. The carrier may also include any agent or solvent with which the constructs of the present invention are compatible and which are non-toxic to the individuals or cells treated at the amounts 10 administered.

Pathological cell proliferative conditions to be treated

There is a wide variety of pathological cancerous and noncancerous cell proliferative conditions for which the method of the present invention will provide therapeutic benefits. 15 These pathological conditions may occur in almost all cell types capable of abnormal cell proliferation. Among the cell types which exhibit pathological or abnormal growth are (1) fibroblasts, (2) vascular endothelial cells and (3) epithelial cells. It can be seen from the above that the method of the 20 present invention is useful in treating local or disseminated pathological conditions in all or almost all organ and tissue systems of the individual.

For instance, in the eye alone, the method of the present invention may be utilized to treat such a wide variety 25 of pathologic disease states caused by abnormal proliferation of normal (benign) or malignant cells or tissues including, but not limited to, the following fibroproliferative, vasoproliferative and/or neoplastic diseases of the eye: retinopathy of prematurity, proliferative vitreoretinopathy, proliferative 30 diabetic retinopathy, capillary hemangioma, choroidal neovascular nets, subretinal neovascular nets, senile macular degeneration due to subretinal, neovascularization, corneal neovascularization, macular pucker due to epiretinal membrane proliferation, adult cataracts due to nuclear sclerosis, fibrous 35 ingrowth following trauma or surgery, optic nerve gliomas, angiomatosis retinae, neovascular glaucoma, cavernous hemangioma, rubeosis iridis, sickle cell proliferative retinopathy,

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epithelial downgrowth after eye surgery or injury, after-cataract membrane, papilloma, retinal neovascularization in thalassemia, subretinal neovascularization due to pseudoxanthoma elasticum, and neurofibromatosis type 1 and 11, retinoblastoma, uveal
5   melanoma and pseudotumor of the orbit. Other benign cell proliferative diseases for which the present invention is useful include, but are not limited to, psoriasis, ichthyosis, papillomas, basal cell carcinomas, squamous cell carcinoma and Stevens-Johnson Syndrome.
10   In general, the present invention is applicable to all forms of cancer including but not limited to cancers in which the inactivation of the Rb gene and/or the p53 gene have been implicated such as osteosarcoma, soft tissue sarcomas, melanomas. fibrosarcoma, retinoblastoma, carcinoma of the breast, bladder,
15   cervix, lung, colon, ovary, kidney, pancreas, and prostate. Furthermore, the novel mutant p53 proteins may be used to induce apoptosis in various cells. Thus, the present invention also is directed to a method of inducing apoptosis comprising the step of administering a novel mutant p53 protein of the present
20   invention.
          In addition, the present invention provides a method of treating a tumor cell having a mutation of a cyclin protein, comprising the step of administering a novel mutant p53 protein of the present invention to a cell. Representative mutations,
25   e.g., translocations, amplifications of cyclin D1, cyclin D2 or cyclin D3 may be treated with the novel mutant p53 proteins of the present invention.

EXAMPLE 1

A conformation determining domain conserved between pRb and p53

30   The present invention discloses a conserved homologous structural domain that controls the conformations of the two tumor suppressor gene products, Rb and p53, thus allowing the creation of mutants of these genes and methods for their use. Thus, the present invention provides an isolated DNA molecule,
35   wherein said molecule is a mutated growth suppressor gene. In one preferred embodiment of the present invention, the mutated growth suppressor gene is a mutated Rb gene. In another

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preferred embodiment of the present invention, the mutated growth
suppressor gene is a mutated P53 gene.

        In an attempt to understand the interaction between pRb
and its cellular targets, antibodies to the various hydrophilic
5   regions of the Rb protein were raised. Since different regions
of the Rb protein may be interacting with different proteins, it
was necessary to raise antibodies to many different regions such
that some of the antibodies would bring down the protein complex
between the Rb protein and its targets without interfering with
10   their interaction. Of a panel of polyclonal anti-Rb antibodies
raised against synthetic peptides with sequences corresponding
to various hydrophilic domains, two of them (Rb1-Ab 18 and Rb1-
Ab20) consistently bring down, in addition to the Rb protein, a
protein of molecular mass 53 kD (Figure 1A, lanes 1, 2, 8 and 9).
15   These two antibodies turned out to be raised against the same
synthetic peptide, p5. The appearance of the 53 kD protein is
specific for antisera raised against p5. Antisera against other
regions, for example Rb1-Ab B (Figure 1, lanes 4-5) and Rb1-Ab
C (Figure 1, lanes 6-7) did not immunoprecipitate the 53 kD
20   protein although they can still recognize the Rb protein.
Because of the similarity in the molecular mass of this protein
and that of p53, it appears that this was the p53 protein itself.
Immunoprecipitation of the cell lines with Pab122, a monoclonal
antibody against p53 showed that the p53 indeed has the same
25   exact molecular mass as that of the 53 kD protein (lane 10).
Furthermore, if p53 was first removed from the cell lysate with
Pab 122, neither Rb1-Ab 18, or Rb1-Ab20 can immunoprecipitate the
53 kD protein, although they still can precipitate the Rb protein
(lane 11). To further verify that the 53 kD protein was indeed
30   p53, immunoprecipitation was done on several cell lines which
expressed no endogenenous p53 and/or Rb protein. In all cases
(lanes 12-20) the antibody indeed can recognize the p53 protein
in accordance with the status of the presence or absence of the
p53   gene   in   these   cell   lines.   Furthermore,   the
35   immunoprecipitation of p53 is independent of the presence or
absence of the Rb protein, suggesting that a complex was not

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formed and that the p53 protein was recognized by the antibodies
directly.

5
To definitively show that the P53 protein shares a
common antigenic domain with the Rb protein, a search of the
amino acid sequence of the p53 protein corresponding to the P5
region of the Rb protein was initiated. If the 3-dimensional
structural information was retained in these two proteins and in
the P5 peptide, there may be a corresponding region on the P53
protein. Furthermore, a peptide with this conserved antigenic
10   domain should block the immunoprecipitation of P53 by RB1-Ab 18
or RB1-Ab 20. Immunoprecipitation of Rb and p53 proteins by Rb1-
Ab18 (Figure 2A) were performed in the presence of various
amounts of these peptides. One such p53 peptide, F19, completely
blocked the immunoprecipitation of p53 by Rb1-Ab18 at the lowest
15   concentration (10 ug/ml) tested (Figure 2A, lanes 12-14). This
was also the same concentration of the Rb peptide p5 used to
block the precipitation (lanes 1 and 2). In contrast, all other
p53 peptides failed to block the immunoprecipitation of p53 by
Rb1-Ab18, even at 50 to 100 times the concentration used (lanes
20   3-11).

The p5 and F19 region are not the only regions that
show structural homology between the Rb and p53 proteins. At
least two other regions were identified by visual and computer
search. For comparison, the homology regions of Rb and p53 are
25   shown in Table I.

TABLE I

Comparison of the homology regions between Rb and p53

(P1 region) (67% homology)

	Rb ───	EINSALVLK	(184-192)
30	P53 ──	ELNEALELK	(343-351)

(P3 region) (67% homology)

	Rb ───	RRGQNRSAR	(245-262)
	P53 ──	KKGQSTSRH	(372-380)

(P5 region) (62% homology)

	Rb ───	KKLRFDIEG--SD	(873-886)
35	P53 ──	KKLMFKTEGPDSD	(381-393)

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Although these regions may appear to be short, those
amino acids that are homologous between pRb and p53 also are the
most evolutionarily conserved ones in p53 (there is no data
available for the sequence of Rb in different species). This
5   high degree of homology between the two growth suppressor
proteins suggest that these regions may have important functions.
A schematic drawing of the positions of the homology regions on
pRb and p53 is shown in Figure 2B. In p53, all three regions are
clustered in the C-terminus of the protein and in pRb regions 1
10   and 3 are clustered in the N-terminal half while the P5 region
is at the C-terminal half. However, in both p53 and pRb these
three regions are all located outside of the domains known to be
important for the suppression function of the proteins. If these
regions are not responsible for the physical interaction with
15   their suppression targets, it may be that they are responsible
for the regulation of the interaction process. Just as it is
important to have active Rb and p53 proteins to suppress
unwarranted cell growth, it is equally important to have a means
to down-regulate their activities so that a cell may proliferate.
20   The presence in these two growth suppressor gene products of the
conserved domains may ensure their coordinated regulation.
Indeed, as is shown below, these homology regions appear to form
a domain which determines the conformations of the two proteins.
Specifically, disruption of this domain by mutation at the
25   conserved amino acids residues (underlined in Table I above) of
any one of these three regions conferred upon the two proteins
an active conformation. In contrast, mutations at the
unconserved amino acids in these regions have no effect.

EXAMPLE 2

30   Figure 10 shows a schematic representation of the
conformations of the wildtype and the mutant p53. Two domains
are described, domain F (function) and domain R (regulation).
Domain F is the region of p53 containing the five domains
conserved in different vertebrate species. Naturally occurring
35   mutation at one of these five domains lead to the inactivation
of the wildtype function of p53. The present invention shows
that domain F is exposed in the Rb/p53 mutant but not in the

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wildtype with important functional consequence. Domain R
represents the C-terminus motif that is just outside of the
domain needed for the binding of p53 to specific DNA sequences.
This domain contains the Rb/p53 homology domain p1, p3, p5.
5   Domain R is hidden in the Rb/p53 mutant but not in the wildtype
with important functional consequences as detailed by the present
invention. Figure 10 shows a schematic diagram of both the wild
type p53 protein showing the domain F and domain R and the mutant
p53 protein showing the domain F and domain R. In Figure 3, the
10 synthetic mutant p53 was shown to bind to specific DNA
sequences, suggesting that the synthetic mutant p53 is assuming
a conformation different from that of the wildtype. Thus, the
DNA binding domain of the synthetic mutant p53 may also be able
to bind to other proteins that can to bind to domain F.
15   EXAMPLE 3

P1, P3, P5 regions control p53 conformation and DNA binding
ability

        The ability of p53 to activate transcription is tightly
related to its ability to bind DNA. Mutant p53 found in cancer
20 cell lines are unable to bind to DNA and unable to activate
transcription. The P53 protein is capable of binding to specific
DNA sequence in the presence of the antibody Pab421. A close
inspection of the epitope of the Pab421 revealed that it overlaps
with the P3 region. The epitope recognized by Pab421 (see Figure
25 3) is the last four amino acid residues in the P3 region. It
appears that blocking this entire P3 region with an antibody
(Pab421) sufficiently changed the conformation of the protein
allowing the p53 protein to bind with a specific DNA sequence.
The DNA binding assay therefore provided a convenient means of
30 testing the role of the P3 and the other homologous regions P1,
and P5 in the control of the conformation of the P53 protein.
As is shown in Figure 3, while the wild type p53 was unable to
bind to its specific DNA sequence (lane 1), it does bind in the
presence of the antibody Pab421 (lane 2). When the conserved
35 amino acids of either the P1 (lane 3), P3 (lanes 5 and 7) or P5
(lane 9) regions of p53 was mutated, the resulting p53 mutant
proteins were able to bind to DNA even in the absence of Pab421.

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The resulting complex was supershifted upon binding to Pab421
(lanes 4, 8, and 10 for P1, P3, P5 respectively). This
supershift is an indication that the P3 region in these mutants
are still capable of binding to the antibodies and that the
5   ability of the mutants to bind DNA in the absence of Pab421 was
not due to mutation at the P3 region. In fact, binding of the
antibodies Pab1801 outside of these C-terminal domain also
allowed a supershift of the complex. The mutant used in lanes
7 and 8 was mutated at the first 4 amino acid residue of the P3
10   region and that apparently does not affect the binding of Pab421
to the protein. The mutant used in lanes 5 and 6 was mutated at
the conserved amino acids of the last 4 residues in the P3 region
and that abolished the binding of Pab421 to the protein.
Mutation at the unconserved amino acids show no effect, the
15   proteins behaved essentially like the wild type. Thus, the
conformation of the mutant p53 proteins is switched to the active
form when the homology regions are destroyed by mutation.

        Examples 3-7 of the present invention demonstrate (1)
the ability of the mutant p53 to bind specific DNA sequences; (2)
20   the ability of the mutant p53 to bind to cyclin D1, D2 and D3;
(3) the ability of the mutant p53 to resist phosphorylation by
the cyclin A/cdk2 kinase at serine residue 315; (4) the ability
of the mutant p53 to activate transcription *in vivo* and (5) the
ability of the synthetic mutant p53 to induce apoptosis of tumor
25   cells when used in conjunction with the chemotherapeutic agent,
cisplatin.

                        EXAMPLE 4
        The present invention shows that p53 is able to bind
to cyclin D1, D2 and D3. The deregulated expression of cyclin
30   D is related to the development of various tumors including
leukemia and mammary tumors. To show the binding of p53 to
cyclin D, the corresponding recombinant baculoviruses were
created and co-infected into sf9 cells. The co-precipitation is
described in Figure 11 which shows that the mutant p53 protein
35   is able to bind specific DNA sequences. The p53 protein was
precipitated using a monoclonal antibody PAb 1801. As is shown
in the Figure 11, wildtype p53 (lane 1) was unable to bind to

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either of the three cyclin Ds when they were coexpressed *in vivo* in infected sf9 cells (lanes 2, 3, 4). In contrast, the mutant form of p53 in which the p3 and p5 regions were deleted (lane 5)
was able to bind to cyclin D1 (lane 2), D2 (lane 3), and D3
5   (lane 4). Thus, the mutation of the Rb/p53 homology regions resulted in the exposure of the functional domain of p53 such that the p53 protein was able to interact with its binding target DNA and proteins. The invention also shows that one such protein was the protooncogene products cyclin D1, 2 and 3, the
10   deregulated expression is important for tumorigenesis.

EXAMPLE 5

In addition to the activation (exposure) of the functional domain of p53, the conformational changes brought about by the mutation of the Rb/p53 region also resulted in
15   specific change in the conformation of domain R in the C-terminus. Specifically, the serine residue 315 was not phosphorylated by any of the cyclin-dependent kinases as the wildtype. Figure 12 shows that the homology regions control the exposure of the C-terminus of p53 so that specific sites are not
20   phosphorylated.

p53 proteins were expressed in bacteria. An aliquot of the bacteria was labeled with ^{35}S-cysteine and methionine for quantitation. Equal amounts of purified p53 (Figure 12B) were mixed with cyclin A: cdk 2 in a phosphorylation buffer containing
25   ^{32}P-ATP. The phosphorylation reactions were carried out at room temperature for 30 minutes. The p53 proteins were then precipitated with PAb1801. As can be seen in figure 12A, p53 was heavily phosphorylated whereas the synthetic mutant P53 mutated at the conserved aminoacid residues in domain P3 (lane 3C) or P5
30   (lane 5C) were not phosphorylated, if at all. In contrast, mutation at the unconserved amino acid residues even within the P3 (3U) or the P5 (5U) regions had no effect on the phosphorylation of the protein. 2-dimensional mapping of the phospho-peptides confirmed that serine 315 is the predominant
35   residue that is phosphorylated.

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EXAMPLE 6

To demonstrate the functional properties of the homology region mutants, the human lung carcinoma cell line H358 which lacks endogenous p53 proteins was cotransfected with the
5   p53 mutants and a reporter construct in which the CAT gene was under the control of a p53 CON. sequence containing a p53 consensus binding site. Figure 13 shows that the homology region mutants of p53 are transcriptionally active *in vivo*. As is shown in Figure 13A, p53 mutants of the P3 or P5 homology region at the
10   conserved aminoacids was active in promoting transcription from a promoter with a p53 binding site and the amount of transcriptional activation by the mutants was equal to or slightly higher than that of the wildtype. In contrast, the p4 mutant (which was mutated in the fifth conserved functional
15   domain) abolished the ability of p53 to bind cyclin D or DNA and was totally defective in promoting transcription. In the middle of Figure 13A, lane 0 represents the control in which only the vector without p53 inset was used. As expected, no transcription promotion can be detected). Equal amount of proteins were used
20   for all the p53 constructs as demonstrated in Figure 13B.

EXAMPLE 7

The introduction of a wildtype p53 has been shown to be effective in the induction of apoptosis of tumor cells when chemotherapeutic agent treatment alone failed to stop tumor
25   growth. To show that the synthetic p53 mutant induced apoptosis, the lung carcinoma cell line H358 which is devoid of endogenous p53 protein due to an inactivating mutation was used. As a comparison, a batch of the same cell line was transfected with either the wildtype or the P4 mutant (which was shown to be
30   inactive by several criteria). Figure 14 shows that the synthetic p53 mutant induces apoptosis in the lung carcinoma cell line, H358. As is shown in Figure 14A, 14C, and 14E, approximately 5% of the H358 cells transfected with wildtype, P4 or P5 mutants respectively showed expression of the p53 proteins
35   in the nucleus by immunostaining with the anti-p53 monoclonal antibody PAb122. (Compare the dark staining of the cell nuclei and the lack of staining in the surrounding cells). After the

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cells were treated with the chemotherapeutic agent c-DDP
(cisplatin) for two weeks, most of the cells that expressed
wildtype (Figures 14B) or p5 mutant p53 (Figures 14F) underwent
·apoptosis and disintegrated. In contrast, cells expressing the
5   p4 inactive mutant p53 (Figure 14D) continued to persist and
indeed proliferated to a greater number than before treatment.
Thus, the present invention demonstrated that the p5 mutant p53
induced apoptosis in conjunction with chemotherapeutic treatment.

EXAMPLE 8

10   Thus, the present invention demonstrates that the
p53/pRb homology regions (P1, P3 and P5) control the
conformations of the p53 protein in two different ways
simultaneously. First, the functional domain (domain F) which
is necessary for cell growth suppression and induction of
15   apotosis, is exposed when the Rb/p53 homology regions in the C-
terminus are modified or mutated. The exposure of the
functionally important central region of these mutant p53
proteins is shown by their enhanced ability to bind to specific
proteins and DNA sequences when compared to the wildtype protein.
20   Secondly, the mutation of the homology regions also brought about
conformational changes at the C-terminal regions. That is,
domain R became hidden such that the ability of p53 to be
phosphorylated at serine #315 is abolished. In addition,
exposure of the C-terminal domains needed for double stranded
25   DNA/RNA annealing activity is controlled by the P3 region, since
blocking the P3 region with PAb421 allows translation of the p53
mRNA. This annealing activity prevents newly synthesized p53
mRNA from being translated. Therefore, mutations at any of the
P1, P3 or P5 regions in the conserved amino acids resulted in a
30   p53 conformation in which various activities known to be
important to the growth suppressor function of the proteins are
unmasked. The conformational change was not specific for any one
particular effect.

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EXAMPLE 9

Alteration in the control of the phosphorylation of Rb proteins mutated at the homology region

There are several ways to distinguish between the
active and inactive conformations of the pRb protein. Active Rb
protein is underphosphorylated and can suppress the growth of the
cell. Inactive Rb protein is heavily phosphorylated. If the
homology domain affects the active and inactive conformation of
pRb, mutation at the P1, P3, P5 regions should affect the ability
of the protein to suppress cell growth and its phosphorylation
accordingly. The P1, P3, and P5 regions of pRb were separately
mutated at the conserved amino acid residues and the ability of
the mutants to suppress cell growth examined. All mutants were
able to suppress the growth of the transfected cells. The
ability of the Rb mutant m89-0, which is mutated at the conserved
amino acid residues at the P5 region, to suppress the growth of
normal and cancer cells is given in two Examples below. Since
the activity of pRb is regulated by phosphorylation, if the
conserved region is for the maintenance of pRb in an inactive
conformation, mutation of the P5 region should have an inhibitory
effect on the phosphorylation of the mutant pRb such that the
protein is active.

There are cell lines which are devoid of pRb expression
due to mutation of both alleles of RB-1 and these cell lines have
also incurred other mutations which render them resistant to the
suppression effect of a transfected Rb gene. The cervical
carcinoma cell line C33A and a subpopulation of the Saos-2 are
two such cell lines. These cell lines therefore would allow the
mutant proteins to be expressed and the cells remain viable,
allowing the extraction of abundant amounts of the protein for
examination of the phosphorylation status. The P5 mutant HuAcPr-
1-neo-P5 C, mutated at the conserved amino acid residues at the
P5 region, was therefore transfected into these cells and stable
cell lines were obtained by single cell purification. The
phosphorylation patterns of the mutant pRb isolated from the
purified mutant cell line Saos-2#89 was compared to that of the
wild type pRb extracted from the wildtype Rb transfected and

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purified cell line Saos-2#84. Immunoprecipitation of pRb from
S phase Saos-2#84 (wildtype) and Saos-2#89 (mutant) showed that
the wildtype pRb was heavily phosphorylated whereas the mutant
pRb was underphosphorylated. This suggests that the P5 mutant
5   pRb remains in the active conformation even when the cells were
in the S phase. Thus, the P5 mutant assumes a conformation which
is resistant to phosphorylation at particular sites. It should
be noted that the protein was still phosphorylated at other
functionally unimportant sites.
10   Since one-dimensional analysis by SDS-PAGE only
revealed that the protein was underphosphorylated but not the
nature of the underphosphorylation, two-dimensional
phosphopeptide analyses of the ^{32}P-labelled pRb using two
different proteases, trypsin and chymotrypsin, was performed.
15 A comparison of the 2-Dimensional maps revealed that there are
2 sites, S10a and S10b, that are consistently unphosphorylated
in the mutant as compare to the wild type (Figure 4A). To show
that the difference in the phosphorylation pattern was due to an
intrinsic difference in the conformation of the proteins instead
20 of variation in *in vivo* enzyme specificity, the *in vitro*
phosphorylation patterns of the proteins were compared. The
wildtype and mutant pRb were phosphorylated using a kinase
preparation that consist of cyclin associated kinases. The *in
vitro* labelling was dependent on the cyclin associated kinase as
25 antibodies against the kinase blocked the phosphorylation. The
in vitro phosphorylated pRb were analyzed by 2-Dimensional
peptide mapping and as shown in Figure 4B, the same
phosphorylation sites were not used in the mutant. Therefore,
even with the supply of abundant amounts of enzymes and optimum
30 phosphorylation conditions in which the wildtype pRB was
phosphorylated to completion, the mutant pRb was completely
resistant to the phosphorylation sites S10a and S10b. The
variation in the phosphorylation of S10a and S10b is therefore
due to an intrinsic difference in the conformation of the
35 wildtype and the mutant proteins instead of variation in *in vivo*
enzyme specificity.

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EXAMPLE 10

Rb proteins mutated at the homology region are active
Correlation of phosphorylation at S10a and S10b with pRb activity

The data provided below demonstrates that the variation
in the phosphorylation at S10a and S10b correlates with the
ability of pRb to bind SV40 large T antigen.   For *in vitro*
phosphorylation studies, Rb protein was isolated from sf9 cells
infected with a recombinant Rb-Baculovirus in which the
polyhedron gene was partially replaced with the Rb gene. It was
noticed that, under high infection titer conditions, although
the majority of the Rb protein extracted was rendered incapable
of binding to the SV40 large T by phosphorylation, a small
proportion of Rb protein that remained bound to large T. This
binding is shown in Figure 5. The Rb protein and the SV 40 large
T were obtained from ^{35}S-labeled SF9 cells infected with the
corresponding bacculoviruses and kinased in vitro. Lane 1 shows
that the Rb proteins extracted consisted of underphosphorylated
as well as hyperphosphorylated forms and some of the proteins
bound   to   large   T.   Large   T   could   bind   only   the
underphosphorylated form of the RB protein (lane 2 and lane 3).
Phosphorylation of this large T:Rb complex with different amounts
of Rb kinase (lanes 3 through 6) released the majority of the Rb
protein from the large T. This form of the RB proteins can be
termed RB-E (E = exposed critical site). However, approximately
20-30% of the Rb protein (termed RB-H) (H = hidden critical site)
remained tightly bound to large T and the whole complex could be
brought   down   by   anti-large   T   antibody.   Most   importantly,
although increasing amount of the Rb kinase could phosphorylate
the Rb protein to its slowest migrating form (compare lane 4
through 6 and lanes 8 through 10), the hyperphosphorylated Rb
protein remained bound to the large T. That the precipitation
of these Rb proteins was not due to nonspecific absorption to
the protein A agarose is shown by comparison of lane 6 with lane
2. Approximately 70% of the Rb protein was released from the
large T. This released Rb protein was not due to a nonspecific
degradation of the protein as shown by the fact that if the
reaction was precipitated with anti-Rb antibodies, all of the Rb

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proteins were accounted for (as shown in lanes 8, 9 and 10). The ability of the various forms of Rb protein to bind to large T is also shown in lanes 8, 9 and 10, in which a smaller amount of large T was brought down by the Rb protein.

5   These results are consistent with the presence of two classes of phosphorylation sites on the Rb protein, with one being critical for the inactivation of the Rb protein so that it cannot bind to large T, whereas the others can be phosphorylated without affecting the binding.   There are 17 potential

10   phosphorylation sites (S/TPXX) for the Rb kinase complex. To demonstrate that the phosphorylation sites are important for the inactivation of the Rb protein, a 2-D mapping analysis was performed on the Rb protein that remains bound to the SV40 large T (RB-H) and on those that are free from the SV40 large T after

15   phosphorylation (RB-E).   There must be a difference in the phosphorylation sites between these two forms of Rb protein if the disassociation of Rb from large T is a result of phosphorylation. As is shown in Figure 6, S10a and S10b were found not to be phosphorylated in the pRb that remain bound to

20   the large T protein.

        Mutation at the conserved amino acids at the P5 region renders the protein an active conformation. The phosphorylation at S10a and S10b is a function of the cell cycle and correlates with the ability of the cell to proliferate.

25   EXAMPLE 11
Phosphorylation at S10a and S10b of pRb and cell growth
        The data provided below demonstrates that the variation in the phosphorylation at S10a and S10b is regulated in a cell cycle dependent manner. It is well documented that exposure of

30   cells, normal or cancerous, to TGF-β frequently lead to growth arrest.   A role of pRb in the suppression of cell growth has previously been described. To show the role of TGF-β on the phosphorylation of pRb, the breast carcinoma cell line MDAMB231 which can be growth arrested by treatment with TGF-β was

35   employed. The proliferation status of the cells were monitored by tritiated thymidine uptake and the phosphorylation pattern of the ^{32}P-labelled pRb analyzed by 2-Dimensional peptide mapping.

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Significantly, the pRb from cells growth arrested by TGF-β were
not phosphorylated at S10a and S10b (Figure 7). However, when
the cells were released from the TGF-β, S10a and S10b were again
phosphorylated. Thus, the pRb protein which is active in growth

5   arrested cells were not phosphorylated at S10a and S10b. That
this reversible phosphorylation was not just specific to the
growth suppression effect of TGF-β was shown by the fact that
similar phosphorylation pattern of S10a and S10b was also found
in pRb isolated from cells growth arrested by deprivation of

10   serum. Upon refeeding with either serum or growth factors, S10a
and S10b was once again phosphorylated. In addition, S10a and
S10b was not phosphorylated in pRb isolated from cells growth
arrested by retinoic acid. Therefore, the functionally critical
S10a and S10b sites in the pRb protein can undergo reversible

15   phosphorylation under different growth conditions.

EXAMPLE 12

Mutants of the Rb gene and the p53 gene

Thus, for both pRb and p53, mutation of the homology
region resulted in the proteins assuming conformations that

20   render them active. Examples of the conformationally active
mutants of Rb and p53, and their uses in suppression of cell
growth, are given below. For the Rb protein, two classes of
mutants are described, (1) mutants in the regulatory or homology
region, and (2) mutants in the phosphorylation sites. For the

25   p53 protein, mutants of the homology regions are described.

Phosphoamino acid analysis of hyperphosphorylated Rb
proteins revealed that the amino acids serine and threonine are
phosphorylated. There are 79 serine and 55 threonine residues
in the Rb protein. Of these, 17 are found in the motif (S/TPXX),

30   a potential phosphorylation site for the cdc2 kinase. The cdc2
kinase, and related kinases, are known to be able to
phosphorylate the Rb protein.

There appear to be two classes of phosphorylation sites
on the Rb protein, with one being critical for the inactivation

35   of the Rb protein so that it cannot bind to large T, whereas the
others can be phosphorylated without affecting the binding.
Because there are at least 17 potential phosphorylation sites

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(S/TPXX) for the Rb kinase complex, several may be important for the inactivation of the Rb protein. These 17 phosphorylation sites are T005, S230, S249, T252, T356, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821, T826, where T and S
5   denotes threonine and serine, respectively, and the number that follows denote the residue number of the amino acid in the Rb protein.

            The mutated retinoblastoma gene is preferably mutated by one of two different ways, both of which affect the ability
10   of the protein to be phosphorylated. First, the gene may be mutated by changing amino acids in conserved homology regions of the Rb gene. Preferably, the conserved homology region of the Rb gene is selected from the group consisting of P1, P3, and P5, as described herein. Representative examples of mutated Rb genes
15   that encodes for a mutated Rb protein is shown in Seq. ID. No. 1 and 2. A person having ordinary skill in this art, with the knowledge of the importance of the conserved homology domains to the phosphorylation of the protein, could easily construct other mutated Rb genes that encode for other desirable mutated Rb
20   proteins.

            A second way of preventing the phosphorylation and thus inactivation of the Rb protein is when said gene is mutated by changing amino acids in the phosphorylation sites. Preferably, the phosphorylation sites are selected from the group consisting
25   of T005, S230, S249, T252, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821 and T826. In the abbreviations for the phosphorylation sites above, S refers to serine, T refers to threonine, and the number refers to the placement of the amino acid in the sequence of the Rb protein. For the phosphorylation
30   mutants disclosed herein, each of the threonine or serine residues was mutated into alanine in individual plasmids. As stated before, there are at least 2 classes of phosphorylation sites, one of which is the functionally important for the inactivation of pRb. Therefore one or more of the mutants
35   created above should negate the phosphorylation of the mutant pRb and prevent its inactivation.

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In other embodiments of the present invention, there are also provided plasmids comprising the DNA encoding for mutated Rb proteins. In addition, also provided is a plasmid adapted for expression in a recombinant cell comprising the DNA

5   encoding for a mutated Rb protein of Seq. ID. No. 1 and regulatory elements necessary for expression of the cDNA in the cell and a plasmid adapted for expression in a recombinant cell comprising the DNA encoding for a mutated Rb protein of Seq. ID. No. 2 and regulatory elements necessary for expression of the

10 cDNA in the cell.

EXAMPLE 12

Mutants of the p53 gene

In another embodiment of the present invention, the mutated growth suppressor gene is a mutated p53 gene.

15 Preferably, the p53 gene is mutated by changing amino acids in conserved homology regions of the p53 gene (underlined in Table I above). Similarly, the conserved homology region of the p53 gene is selected from the group consisting of P1, P3 and P5. Mutants can be consisting of mutations at either P1, P3, or P5,

20 singularly or in any combinations (including mutations of all three regions). In addition, mutants in which the C-terminal amino acid residues 295-393 were deleted were still active in binding to the specific DNA sequence.

EXAMPLE 13

25 ### Use of pRb and p53 mutants for cell growth suppression

The present invention also discloses novel transfected cells, e.g., the transfected cell comprising the isolated DNA molecule of Claim 1 which encodes a mutated Rb protein. Also provided by the present invention are stably transfected cells

30 expressing a mutated Rb gene encoding for a mutated Rb protein. Preferably, the transfected cells of present invention contain the mutated Rb gene comprising a nucleic acid sequence encoding for a mutated Rb protein shown in Seq. ID No. 1 or Seq. ID No. 2. In addition, the present invention also provides a stably

35 transfected cell expressing a mutated p53 gene encoding for a mutated p53 protein.

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The present invention also provides a method for the treatment of pathological cell proliferative diseases comprising administration to a non-cancerous proliferating cell of the DNA molecule of a mutated growth suppressor gene. The molecule

5   administered, in one embodiment, is a mutated Rb or p53 gene. The gene is mutated by changing amino acids in conserved homology regions of the Rb gene or said gene is mutated by changing amino acids in phosphorylation sites. The conserved homology regions and the phosphorylation sites are the same as described above.

10 Generally, this method may be used to treat any cell proliferative disease. Representative examples of non-cancerous cell proliferative disease include psoriasis, benign proliferative skin diseases, ichthyosis, papilloma, basal cell carcinoma, squamous cell carcinoma, fibroproliferative diseases,

15 vasoproliferative diseases and dermatoproliferative diseases.

The present invention also provides a method of treating malignant cell diseases in individuals comprising administration into a proliferating cancer cell of a DNA molecule of a mutated Rb or p53 gene. These mutated genes are constructed

20 as described above.

Generally, the method of treating malignant cell diseases is similar to the treatment of non-cancerous cell proliferative diseases. Representative examples of diseases caused by proliferating cancer cells, include but not limited to,

25 osteosarcoma, fibrosarcoma, retinoblastoma, carcinoma of the breast, bladder, cervix, lung, colon, ovary, kidney, pancreas, and prostate.

According to the method of the present invention, one can manipulate the cellular proliferative process by the

30 introduction of a mutant RB or p53 protein to prevent or inhibit abnormal proliferation in a wide variety of cell proliferative diseases. The manipulation of the proliferative process may be accomplished by introducing into a target proliferating cell a DNA construct which encodes a mutant RB or p53 protein element.

35 The present invention also provides a method for the treatment of focal cell proliferative diseases comprising administration of the mutated Rb or p53 gene to proliferating

cells. Generally, the proliferating cell may be the cause of such representative diseases as fibroproliferative, vasoproliferative and neoplastic diseases in the eye.

5   In another embodiment, the phosphorylation site of the Rb gene is mutagenized before introduction into a cell. There are 17 potential phosphorylation sites on the Rb protein. Mutation of the serine or threonine coding sequences in the Rb cDNA into alanine or valine or others would therefore lead to the production of a permanently active Rb protein which cannot be

10   inactivated by phosphorylation. That is, the host cell will not be able to inactivate the Rb protein by phosphorylation. Introduction of such a mutated Rb gene into a cell will therefore lead to growth arrest. All these mutants have been subcloned in the human β-actin promoter vector. For subcloning of the Rb

15   cDNA, the entire open reading frame was ligated to a synthetic linker containing the BamH1 site at the 5' end and a synthetic linker containing AATAAA and a BamH1 site at the 3' end. The resulting BamH 1 fragment was subcloned into the BamH 1 site of the vector and the clone with the correct orientation selected.

20   EXAMPLE 14

Preparation of the p5 mutants m89-0 and m88-0

To create the m89-0 mutant, a pair of DNA oligonucleotide primers (Pm1 and QM1) containing the region 'P5' to be mutated and a pair of oligonucleotides flanking the region

25   'P5' (P3 and Q3) were synthesized using the AB1 PCR-mate oligonucleotide synthesizer according to manufacturer instruction. Using the wild type Rb cDNA as the template, 40 cycles of polymerase chain reactions (PCR) were performed with the primers P3 and Qm1. In a second tube, 40 cycles of

30   polymerase chain reactions were performed with the primers Pm1 and Q3. After the completion of the reaction, a small aliquot of the reaction product was taken from the first tube and 80 cycles of PCR were performed in the presence of excess P3 primer. Similarly, 80 cycles of PCR w re performed in the presence of

35   excess Q3 primer in the second tube. The predominately single strand reaction products from these two tubes were then combined and allowed to primer extend for 20 cycles. The resulting double

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stranded product, now containing the mutated P5 region were
subcloned into the Rb expression plasmid HuBAcpr-1-neo-PQ (m89-0)
at the Mlu 1 and Hind 3 sites. The resulting plasmid has a
mutated P5 region shown below with the mutated amino acids
5   underlined. K870 and H890 refer to the lysine and histidine
amino acid residues at position 870 and 890 respectively in the
Rb protein. m88-0 was created similarly.

Wildtype   Rb:- K_{870}PLKKLRFDIEGSDEADGSKH$_{890}$
     Mutant m89-0 Rb:- K_{870}PLKKLRFDIEASAEVDASIH$_{890}$
10   Mutant m88-0 Rb:- K_{870}LLIKPRYDTEGSDEADGSKH$_{890}$

EXAMPLE 15
Effect of Transfected Plasmids on the Human Fibroblast Cell Line
          The effect of retinoblastoma cDNA on cell growth was
seen as the mutant m89-0 was transfected into the normal human
15   fibroblast cell line WS1. The gene for the SV40 large T antigen
was used as a control for the transfection. A plasmid, pPVUO-
Neo, which contains the gene for the SV40 large T antigen and a
neomycin resistance gene was then transfected in order to monitor
the efficiency of transfection. In addition, an inactive mutant
20   form of the Rb gene, "HuBAcpr-1-neo-P16" was used as a control.
For transfection, 100 μg of each of the plasmid DNA were mixed
with 107 exponentially grown WS1 cells in a final volume of 0.8
ml of RPM1 1640 medium (Gibco) plus 10% FCS in an electroporation
chamber unit Cell-PoratorTM (BRL). Electroporation was done at
25   200 volt and 1180 μF. The electroporated cells were allowed to
recover at room temperature for 10 minutes and were then diluted
in RPM1 1640 plus 10% fetal calf serum (FCS) and plated out in
60 mm dishes at a cell density of 2 X 104 cells/cm2. The cells
were allowed to attach and grow in the same medium for two days.
30   Thereafter, the medium was changed to RPM1 1640 + 10% FCS +
15ug/ml G418 (GIBCO). Every three days, duplicate dishes were
taken for histoimmunochemical staining using either a rabbit
polyclonal anti-Rb protein antibodies RB1-ABA1 (Figure 8A), or
with the mouse monoclonal anti-SV40 large T antigen antibody Pab
35   101 (Figure 8B). As can be seen from Figure 8A, 13 days after

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transfection and selection in G418 medium, the WS1 cells
expressing the transfected Rb cDNA plasmid m89-0 stained
intensely with the anti-Rb protein antibodies. However, the
cells that expressed the Rb protein remained as single cells and
5   did not divide. In contrast, cells that expressed the
transfected SV40 large T antigen (SVLT) continued to divide into
colonies as shown by the group of cells stained positive with the
mouse anti SVLT antibody in Figure 8B. Thus, over expression of
the m89-0 Rb cDNA in a cell led to complete arrest of cell growth
10   of the normal cells WS1. Cells transfected with the inactive
form of Rb HuβAcpr-1-neo-P16 were not suppressed but divided into
colonies.

EXAMPLE 16

Effect of Transfected Plasmids on a Bladder Carcinoma Cell Line

15   The mutant m89-0 and HuβAcpr-1-neo-P16 plasmids were
also transfected into the human bladder carcinoma cells TCCSUP
which has no endogenous Rb protein using the same procedures
described above. The plasmids used, the methodology for
transfection and immunostaining are identical to those described
20   above.

As can be seen in Figure 9A, cells expressing the
transfected m89-0 cDNA failed to divide. In contrast, cells
expressing the transfected HuBAcpr-1-neo-P16 divided to form
colonies (Figure 9B). Overexpression of the m89-0 Rb cDNA led
25   to severe retardation of growth of the tumor cells *in vitro*.

EXAMPLE 17

In addition to the mutants at the regions homologous
between Rb and p53, mutations in the p53 protein. Table II lists
the partial amino acid sequence of the wild type p53 and
30   conserved amino acid mutants prepared. In addition, Table II
lists the DNA sequence corresponding to the mutant amino acid
sequence. To prepare the mutants, two primers were synthesized
and annealed together. PCR was carried out as described above.
As shown in above, the alteration P1, P3 and P5 regions 1 ad to
35   a change in the conformation of the p53 protein such that it is
now capable of binding to its target DNA sequence directly.

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TABLE II

<u>p53 MUTANTS</u>

    P1   WIldTYPE: ELNEALELK

      CONSERVED a.a. MUTANT (P53-Con P1): KLYEDFEIE

5   (1) 5' GC TCC GAG ATG TTC CGA AAG CTG TAT GAG GAC TTG GAA ATC GAG GAT GCC CAG GCT GGG 3'

   (2) 3' CG AGG CTC TAC AAG GCT TTC GAC ATA CTC CTG AAC CTT TAG CTC CTA CGG GTC CGA CCC 5'

    P3   WILDTYPE: KKGQSTSRH

10   CONSERVED aa MUTANT (P53-Con P3): NEWFSTARD

   (1) 5' AGC CAC CTG AAG TCC AAC GAG TGG TTC TCT ACC GCC CGC GAT AAA AAA CTC ATG TTC AAG AC 3'

   (2) 3' TCG GTG GAC TTC AGG TTG CTC AGG AAG AGA TGG CGG GCG CTA TTT TTT GAG TAC AAG TTC TG 5'

15 P5   WILDTYPE: KKLMFKTEGPDSD

      Conserved a.a. MUTANT (P53-Con P5): NEVMWKTKWPDAH

   (1) 5'GTCTACCTCCCGCCAT AACGAA GTCATG TGGAAG ACAAAA TGGCCT GACGCA CAC TGACATTCTCCACTTCTT 3'

   (2) 3'CAGATGGAGGGCGGTA TTGCTT CAGTAC ACCTTC TGTTTT ACCGGA CTGCGT

20 GTG ACTGTAAGAGGTGAAGAA 5'

EXAMPLE 18

<u>Generation of the recombinant retrovirus containing a mutant Rb</u>
<u>gene</u>

      A Hind III/Bam HI fragment containing the entire coding

25 region of the mutant Rb#89 is subcloned into the Hind III/BgIII
sites in a retroviral vector. The resulting plasmid vectors are
transfected into the cell line Psi-2. The medium used to culture
the transfected cells is infected into another cell line PA317.
Cells that produce the recombinant virus were selected in G418

30 and the retrovirus collected from the medium is concentrated and
purified through a sucrose gradient. Mutants of the p53 gene is
tested in an identical manner.

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EXAMPLE 19

Effect of the G1NaSvRb89 virus on cell proliferation in the eyes

Experimental Proliferative Vitreous Retinopathy (PVR) in rabbits is induced by injection of rabbit fibroblasts and
5   induction of local wound. This procedure consists of inducing a wound at limbus and injecting 2×10^5 rabbit fibroblasts into the vitreous under general anesthesia. Post-operatively, the rabbit will receive antibiotic containing local ointment.

Recombinant retroviruses with the mutant Rb or p53 gene
10   are injected into the vitreous (2×10^5 cells/rabbit) with the animal under general anesthesia. Initially, local tolerance of viruses is tested by assessing the maximum dosage of virus (concentration of the virus to be used and the volume of injecting virus). Retroviruses have already been used for
15   injection beneath the retina in mice without reported side effects (Turner and Cepko, *Nature* 328:132, 1987). The maximum dosage that would not cause side effects will be employed for all subsequent treatment.

The growth of cells *in vivo* is monitored by
20   photography. The grade of retina detachment is compared with the controls where only a retrovirus containing a 1-Galactosidase gene was injected. Additionally, the growth of cells *in vivo* is monitored by labelling intra-vitum with BromodeoxyUridine. One hour before euthanasia, the rabbit is given 50 mg/kg BrdU I.V.
25   to label the proliferating cells. After euthanasia, the cells are analyzed for BrdU incorporation into their DNA by immunostaining with antibodies to BrdU. Simultaneously, immunostaining of the cells with antibodies against the Rb or p53 protein or the 1-Galactosidase protein are also performed to
30   assess the efficiency of the infection.

The optimum time for the retrovirus to suppress the growth of the injected cells is assessed by injecting the virus at various time after induction of PVR. The effect of the vitreous on the infection efficiency in rabbit fibroblasts is
35   also determined. The time delay for adding these viruses on rabbit fibroblasts is also determined. The time delay for adding these viruses on rabbit fibroblasts that stimulated with

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fibroblast growth factors to increase the growth rate is also determined.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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<u>SEQUENCE LISTING</u>

(1) GENERAL INFORMATION:

    (i) APPLICANT: Fung, Yuen Kai

    (ii) TITLE OF INVENTION: Mutants of the Rb and p53 Genes
                                   and Uses Thereof

    (iii) NUMBER OF SEQUENCES: 15

    (iv) CORRESPONDENCE ADDRESS:

        (A) ADDRESSEE: James F. Weiler, Attorney-at-Law

        (B) STREET: One Riverway, Suite 1560

        (C) CITY: Houston

        (D) STATE: Texas

        (E) COUNTRY: USA

        (F) ZIP: 77056

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: DS, HD 1.44 Mb/1.44 Mo

        (B) COMPUTER: IBM PC compatible

        (C) OPERATING SYSTEM: PC-DOS/MS-DOS

        (D) SOFTWARE: WordPerfect 6.0

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER:

        (B) FILING DATE:

        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:

        (A) NAME: Weiler, James F.

        (B) REGISTRATION NUMBER: 16,040

        (C) REFERENCE/DOCKET NUMBER: D-5382 CIP

    (ix) TELECOMMUNICATION INFORMATION:

        (A) TELEPHONE: 713-626-8646

        (B) TELEFAX: 713-963-5853

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: amino acid

        (C) STRANDEDNESS:

        (D) TOPOLOGY: linear

-40-

(ii) MOLECULE TYPE:

    (A) Description: peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v) FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE:

    (B) STRAIN:

    (C) INDIVIDUAL ISOLATE:

    (D) DEVELOPMENTAL STAGE:

    (F) TISSUE TYPE:

    (G) CELL TYPE:

    (H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Leu Leu Ile Lys Pro Arg Tyr Asp Thr Glu Gly Ser Asp Glu

    1   5   10

    Ala Asp Gly Ser Lys His

    15   20

(3) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 20

        (B) TYPE: amino acid

        (C) STRANDEDNESS:

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: peptide

    (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

    (v) FRAGMENT TYPE: internal fragment

    (vi) ORIGINAL SOURCE:

        (B) STRAIN:

        (C) INDIVIDUAL ISOLATE:

        (D) DEVELOPMENTAL STAGE:

        (F) TISSUE TYPE:

        (G) CELL TYPE:

        (H) CELL LINE:

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```
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
     Pro Leu Lys Lys Leu Arg Phe Asp Ile Glu Ala Ser Ala Glu
      1                   5                        10
     Val Asp Ala Ser Ile His
          15                  20
```

(4) INFORMATION FOR SEQ ID NO:3:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 20

      (B) TYPE: amino acid

      (C) STRANDEDNESS:

      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE:

      (A) Description: peptide

   (iii) HYPOTHETICAL: No

   (iv) ANTISENSE: No

   (v) FRAGMENT TYPE: internal fragment

   (vi) ORIGINAL SOURCE:

      (B) STRAIN:

      (C) INDIVIDUAL ISOLATE:

      (D) DEVELOPMENTAL STAGE:

      (F) TISSUE TYPE:

      (G) CELL TYPE:

      (H) CELL LINE:

   (ix) FEATURE:

      (A) OTHER INFORMATION: Represents amino acids 871-890 of the retinoblastoma protein.

```
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:
     Pro Leu Lys Lys Leu Arg Phe Asp Ile Glu Gly Ser Asp Glu
      1                   5                        10
     Ala Asp Gly Ser Lys His
          15                  20
```

(5) INFORMATION FOR SEQ ID NO:4:

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(i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 9
        (B) TYPE: amino acid
        (C) STRANDEDNESS:
5   (D) TOPOLOGY: linear
(ii) MOLECULE TYPE:
        (A) Description: peptide
(iii) HYPOTHETICAL: No
(iv) ANTISENSE: No
10 (v) FRAGMENT TYPE: internal fragment
(vi) ORIGINAL SOURCE:
        (B) STRAIN:
        (C) INDIVIDUAL ISOLATE:
        (D) DEVELOPMENTAL STAGE:
15   (F) TISSUE TYPE:
        (G) CELL TYPE:
        (H) CELL LINE:
(ix) FEATURE:
        (A) OTHER INFORMATION: Represents amino acids 184-
20   192 of the retinoblastoma
                                 protein.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:
        Glu Ile Asn Ser Ala Leu Val Leu Lys
        1   5

25 (6) INFORMATION FOR SEQ ID NO:5:
        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 9
                (B) TYPE: amino acid
                (C) STRANDEDNESS:
30   (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE:
                (A) Description: peptide
        (iii) HYPOTHETICAL: No
        (iv) ANTISENSE: No
35   (v) FRAGMENT TYPE: internal fragment

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(vi) ORIGINAL SOURCE:

    (B) STRAIN:

    (C) INDIVIDUAL ISOLATE:

    (D) DEVELOPMENTAL STAGE:

5   (F) TISSUE TYPE:

    (G) CELL TYPE:

    (H) CELL LINE:

(ix) FEATURE:

    (A) OTHER INFORMATION: Represents amino acids 343-

10   351 of the p53 protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

    Glu Leu Asn Glu Ala Leu Glu Leu Lys

    1   5

(7) INFORMATION FOR SEQ ID NO:6:

15   (i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 9

    (B) TYPE: amino acid

    (C) STRANDEDNESS:

    (D) TOPOLOGY: linear

20   (ii) MOLECULE TYPE:

    (A) Description: peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v)   FRAGMENT TYPE: internal fragment

25   (vi) ORIGINAL SOURCE:

    (B) STRAIN:

    (C) INDIVIDUAL ISOLATE:

    (D) DEVELOPMENTAL STAGE:

    (F) TISSUE TYPE:

30   (G) CELL TYPE:

    (H) CELL LINE:

(ix) FEATURE:

    (A) OTHER INFORMATION: Represents amino acids 245-

                                       262 of the retinoblastoma

35   protein.

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        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
             Arg Arg Gly Gln Asn Arg Ser Ala Arg
             1   5

     (8) INFORMATION FOR SEQ ID NO:7:
 5   (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 9
                (B) TYPE: amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY: linear
10   (ii) MOLECULE TYPE:
                (A) Description: peptide
          (iii) HYPOTHETICAL: No
           (iv) ANTISENSE: No
            (v) FRAGMENT TYPE: internal fragment
15   (vi) ORIGINAL SOURCE:
                (B) STRAIN:
                (C) INDIVIDUAL ISOLATE:
                (D) DEVELOPMENTAL STAGE:
                (F) TISSUE TYPE:
20   (G) CELL TYPE:
                (H) CELL LINE:
           (ix) FEATURE:
                (A) OTHER INFORMATION: Represents amino acids 372-
                                         380 of the p53 protein.
25   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
                Lys Lys Gly Gln Ser Thr Ser Arg His
                1   5

     (9) INFORMATION FOR SEQ ID NO:8:
            (i) SEQUENCE CHARACTERISTICS:
30   (A) LENGTH: 14
                (B) TYPE: amino acid
                (C) STRANDEDNESS:
                (D) TOPOLOGY: linear
           (ii) MOLECULE TYPE:
35   (A) Description: peptide

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(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v)   FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE:

     (B) STRAIN:

     (C) INDIVIDUAL ISOLATE:

     (D) DEVELOPMENTAL STAGE:

     (F) TISSUE TYPE:

     (G) CELL TYPE:

     (H) CELL LINE:

(ix) FEATURE:

     (A) OTHER INFORMATION: Represents amino acids 873-886 of the retinoblastoma protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Lys Leu Arg Phe Asp Ile Glu Gly Ser Asp Glu Ala Asp
1   5   10

(10) INFORMATION FOR SEQ ID NO:9:

(i)   SEQUENCE CHARACTERISTICS:

     (A) LENGTH: 13

     (B) TYPE: amino acid

     (C) STRANDEDNESS:

     (D) TOPOLOGY: linear

(ii) MOLECULE TYPE:

     (A) Description: peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

(v)   FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE:

     (B) STRAIN:

     (C) INDIVIDUAL ISOLATE:

     (D) DEVELOPMENTAL STAGE:

     (F) TISSUE TYPE:

     (G) CELL TYPE:

     (H) CELL LINE:

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(ix) FEATURE:

    (A) OTHER INFORMATION: Represents amino acids 381-393 of the p53 protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    Lys Lys Leu Met Phe Lys Thr Glu Gly Pro Asp Ser Asp

    1   5   10

(11) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 9

        (B) TYPE: amino acid

        (C) STRANDEDNESS:

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: peptide

    (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

    (v) FRAGMENT TYPE: internal fragment

    (vi) ORIGINAL SOURCE:

        (B) STRAIN:

        (C) INDIVIDUAL ISOLATE:

        (D) DEVELOPMENTAL STAGE:

        (F) TISSUE TYPE:

        (G) CELL TYPE:

        (H) CELL LINE:

    (ix) FEATURE:

        (A) OTHER INFORMATION: Represents mutant protein of amino acids 343-351 of the p53 protein.

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

    Lys Leu Tyr Glu Asp Phe Glu Ile Glu

    1   5

(12) INFORMATION FOR SEQ ID NO.11:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 59

        (B) TYPE: nucleic acid

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        (C) STRANDEDNESS: double stranded
        (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE:
        (A) Description: other
5   (iii) HYPOTHETICAL: No
    (iv) ANTISENSE: No
    (vi) ORIGINAL SOURCE:
        (B) STRAIN:
        (C) INDIVIDUAL ISOLATE:
10   (D) DEVELOPMENTAL STAGE:
        (F) TISSUE TYPE:
        (G) CELL TYPE:
        (H) CELL LINE:
    (ix) FEATURE:
15   (A) OTHER INFORMATION: Represents   nucleic   acids
                                encoding mutant protein of
                                sequence ID No. 10.
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO.11:
    GCTCCGAGAT GTTCCGAAAG CTGTATGAGG ACTTGGAAAT CGAGGATCGG   50
20 CAGGCTGGG   59

    (13) INFORMATION FOR SEQ ID NO:12:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 9
            (B) TYPE: amino acid
25   (C) STRANDEDNESS:
            (D) TOPOLOGY: linear
        (ii) MOLECULE TYPE:
            (A) Description: peptide
        (iii) HYPOTHETICAL: No
30   (iv) ANTISENSE: No
        (v) FRAGMENT TYPE: internal fragment
        (vi) ORIGINAL SOURCE:
            (B) STRAIN:
            (C) INDIVIDUAL ISOLATE:
35   (D) DEVELOPMENTAL STAGE:
            (F) TISSUE TYPE:

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```
                    (G)  CELL TYPE:
                    (H)  CELL LINE:
              (ix)  FEATURE:
                    (A)  OTHER INFORMATION:    Represents mutant protein of
    5                                          amino  acids  372-380  of  the
                                               p53 protein.
              (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
                    Asn Glu Trp Phe Ser Thr Ala Arg Asp
                    1                   5


   10    (14) INFORMATION FOR SEQ ID NO.13:
              (i)  SEQUENCE CHARACTERISTICS:
                    (A)  LENGTH: 62
                    (B)  TYPE: nucleic acid
                    (C)  STRANDEDNESS: double stranded
   15               (D)  TOPOLOGY: linear
              (ii) MOLECULE TYPE:
                    (A)  Description: other
              (iii) HYPOTHETICAL: No
              (iv)  ANTISENSE: No
   20         (vi) ORIGINAL SOURCE:
                    (B)  STRAIN:
                    (C)  INDIVIDUAL ISOLATE:
                    (D)  DEVELOPMENTAL STAGE:
                    (F)  TISSUE TYPE:
   25               (G)  CELL TYPE:
                    (H)  CELL LINE:
              (ix)  FEATURE:
                    (A)  OTHER INFORMATION:    Represents   nucleic   acids
                                               encoding  mutant  protein  of
   30                                          sequence ID No. 12.
              (xi) SEQUENCE DESCRIPTION: SEQ ID NO.13:
              AGCCACCTGA AGTCCAACGA GTGGTTCTCT ACCGCCCGCG ATAAAAAACT   50
              CATGTTCAAG AC   62


       (15) INFORMATION FOR SEQ ID NO:14:
```

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(i)   SEQUENCE CHARACTERISTICS:

        (A)   LENGTH: 13

        (B)   TYPE: amino acid

        (C)   STRANDEDNESS:

5   (D)   TOPOLOGY: linear

(ii) MOLECULE TYPE:

        (A)   Description: peptide

(iii) HYPOTHETICAL: No

(iv) ANTISENSE: No

10 (v)   FRAGMENT TYPE: internal fragment

(vi) ORIGINAL SOURCE:

        (B)   STRAIN:

        (C)   INDIVIDUAL ISOLATE:

        (D)   DEVELOPMENTAL STAGE:

15   (F)   TISSUE TYPE:

        (G)   CELL TYPE:

        (H)   CELL LINE:

(ix) FEATURE:

        (A)   OTHER INFORMATION: Represents mutant protein of

20   amino acids 381-393 of the

                                   p53 protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

        Asn Glu Val Met Trp Lys Thr Lys Trp Pro Asp Ala His

        1   5   10

25 (16) INFORMATION FOR SEQ ID NO.15:

        (i)   SEQUENCE CHARACTERISTICS:

                (A)   LENGTH: 73

                (B)   TYPE: nucleic acid

                (C)   STRANDEDNESS: double stranded

30   (D)   TOPOLOGY: linear

        (ii) MOLECULE TYPE:

                (A)   Description: other

        (iii) HYPOTHETICAL: No

        (iv) ANTISENSE: No

35   (vi) ORIGINAL SOURCE:

                (B)   STRAIN:

-50-

         (C) INDIVIDUAL ISOLATE:

         (D) DEVELOPMENTAL STAGE:

         (F) TISSUE TYPE:

         (G) CELL TYPE:

5   (H) CELL LINE:

    (ix) FEATURE:

         (A) OTHER INFORMATION: Represents   nucleic   acids
                        encoding mutant protein of
                        sequence ID No. 14.

10   (xi) SEQUENCE DESCRIPTION: SEQ ID NO.15:

GTCTACCTCC CGCCATAACG AAGTCATGTG GAAGACAAAA TGGCCTGACG 50

CACACTGACA TTCTCCACTT CTT 73

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Claims

1.   An isolated DNA molecule, wherein said molecule
is a synthetic mutated Rb gene encoding a mutated functionally
active retinoblastoma protein.

5   2.   The molecule of claim 1, wherein said gene is
mutated by changing nucleotides that encode for amino acids in
conserved homology regions of the Rb gene.

3.   The molecule of claim 2, wherein said conserved
homology region of the Rb protein is selected from the group
10   consisting of P1, P3 and P5.

4.   The molecule of Claim 1, wherein said gene encodes
an amino acid sequence selected from the group consisting of the
sequences shown in Seq. ID. Nos. 1 and 2.

5.   The molecule of claim 1, wherein said gene is
15   mutated by changing the nucleotide sequence that encodes amino
acids in phosphorylation sites of the Rb protein.

6.   The   molecule   of   claim   5,   wherein   said
phosphorylation sites are selected from the group consisting of
T005, S230, S249, T252, T373, S567, S608,   S612, T773, S780,
20   S788, S795, S807, S811, T821 and T826.

7.   An isolated DNA molecule, wherein said molecule
is a synthetic mutated p53 gene encoding a mutated functionally
active p53 protein.

8.   The molecule of claim 7, wherein said gene is
25   mutated by changing the nucleotide sequence that encodes for
amino acids in conserved homology regions of the p53 protein.

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9.   The molecule of claim 8, wherein said conserved homology region of the p53 protein is selected from the group consisting of P1, P3 and P5.

10.   A plasmid comprising the DNA of Claim 1.

11.   A plasmid comprising the DNA of Claim 7.

12.   A plasmid adapted for expression in a recombinant cell comprising cDNA encoding for the amino acid sequence of Seq. ID. No. 1 and regulatory elements necessary for expression of the cDNA in the cell.

13.   A plasmid adapted for expression in a recombinant cell comprising cDNA encoding for the amino acid sequence of Seq. ID. No. 2 and regulatory elements necessary for expression of the cDNA in the cell.

14.   A method for the treatment of pathological cell proliferative diseases comprising administration to a non-cancerous proliferating cell of the DNA molecule of claim 1.

15.   The method of claim 14, wherein said gene is mutated by changing the nucleotide sequence that encodes amino acids in conserved homology regions of the Rb gene.

16.   The method of claim 15, wherein said conserved homology region of the Rb gene is selected from the group consisting of P1, P3 and P5.

17.   The method of Claim 16, wherein said gene encodes an amino acid sequence selected from the sequences shown in Seq. ID. No. 1 and 2.

18.   The method of claim 14, wherein said gene is mutated by changing the nucleotide sequence that encodes amino acids in phosphorylation sites of the Rb protein.

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19. The method of claim 18, wherein said phosphorylation sites are selected from the group consisting of T005, S230, S249, T252, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821 and T826.

20. A method for the treatment of pathological cell proliferative diseases comprising administration to a non-cancerous proliferating cell of the DNA molecule of claim 7.

21. The method of claim 20, wherein said gene is mutated by changing the nucleotide sequence encoding amino acids in conserved homology regions of the p53 protein.

22. The method of claim 21, wherein said conserved homology region of the p53 protein is selected from the group consisting of P1, P3 and P5.

23. The method of claim 20, wherein said non-cancerous proliferating cell disease is selected from the group consisting of psoriasis, benign proliferative skin diseases, ichthyosis, papilloma, basal cell carcinoma, squamous cell carcinoma, fibroproliferative diseases, vasoproliferative diseases and dermatoproliferative diseases.

24. A method of treating malignant cell diseases in individuals comprising administration into a proliferating cancer cell of a DNA molecule of claim 1.

25. The method of claim 24, wherein said gene is mutated by changing the nucleotide sequence encoding amino acids in conserved homology regions of the Rb gene.

26. The method of claim 25, wherein said conserved homology region of the Rb gene is selected from the group consisting of P1, P3 and P5.

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27. The method of Claim 24, wherein said gene encodes an amino acid sequence selected from the sequences shown in Seq. ID. No. 1 and 2.

28. The method of claim 24, wherein said gene is mutated by changing the nucleotide sequence encoding amino acids in phosphorylation sites of the Rb protein.

29. The method of claim 28, wherein said phosphorylation sites are selected from the group consisting of T005, S230, S249, T252, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821 and T826.

30. A method of treating malignant cell diseases in individuals comprising administration into a proliferating cancer cell of a DNA molecule of claim 7.

31. The method of claim 30, wherein said gene is mutated by changing the nucleotide sequence encoding amino acids in conserved homology regions of the p53 protein.

32. The method of claim 31, wherein said conserved homology region of the p53 protein is selected from the group consisting of P1, P3 and P5.

33. The method of claim 30, wherein said proliferating cancer cell is a cause of a disease selected from the group consisting of ovarian cancer, bladder cancer, lung cancer, cervical cancer, breast cancer, prostate cancer, glioma, fibrosarcoma, retinoblastoma, melanomas, soft tissue sarcomas, osteosarcoma, colon cancer, carcinoma of the kidney and pancreatic cancer.

34. A method for the treatment of focal cell proliferative diseases comprising administration of a mutated Rb gene of claim 1 to proliferating cells.

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35. The method of Claim 34, wherein said proliferating cell is the cause of a disease selected from the group consisting of fibroproliferative, vasoproliferative and neoplastic diseases in the eye.

5   36. A method for the treatment of focal cell proliferative diseases comprising administration of the mutated p53 gene of claim 7 to proliferating cells.

37. The method of Claim 36, wherein said proliferating cell is the cause of a disease selected from the group consisting 10   of fibroproliferative, vasoproliferative and neoplastic diseases in the eye.

38. A mutated retinoblastoma protein produced by the gene of claim 1.

39. A mutated P53 protein produced by the gene of 15   claim 7.

40. A method for the treatment of pathological cell proliferative diseases comprising administration concurrently or consecutively to a non-cancerous proliferating cell a mutated Rb gene and a mutated P53 gene.

20   41. A method of treating malignant cell diseases in individuals comprising administration concurrently or consecutively into a proliferating cancer cell of a mutated Rb gene and a mutated P53 gene.

42. A method of inducing apoptosis, comprising the 25   step of administering the protein of claim 39 to a cell.

43. A method of treating a tumor cell having a mutation of a cyclin protein, comprising the step of administering the protein of claim 39 to a cell.

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44.   The method of claim 43, whether said cyclin is selected from the group of cyclin D1, cyclin D2 and cyclin D3.

FIG. 2A

FIG. 1

FIG. 2B

1 2 3 4 5 6 7 8 9 10

FIG. 3

1   2   3   4   1   2   3   4

−Rb

−LT

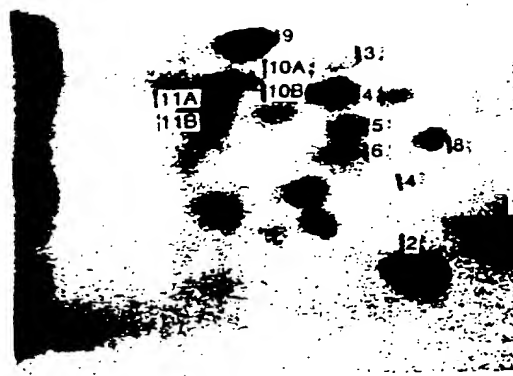FIG. 5A   FIG. 5B

FIG. 4A

FIG. 4B

FIG. 4C

FIG. 4D

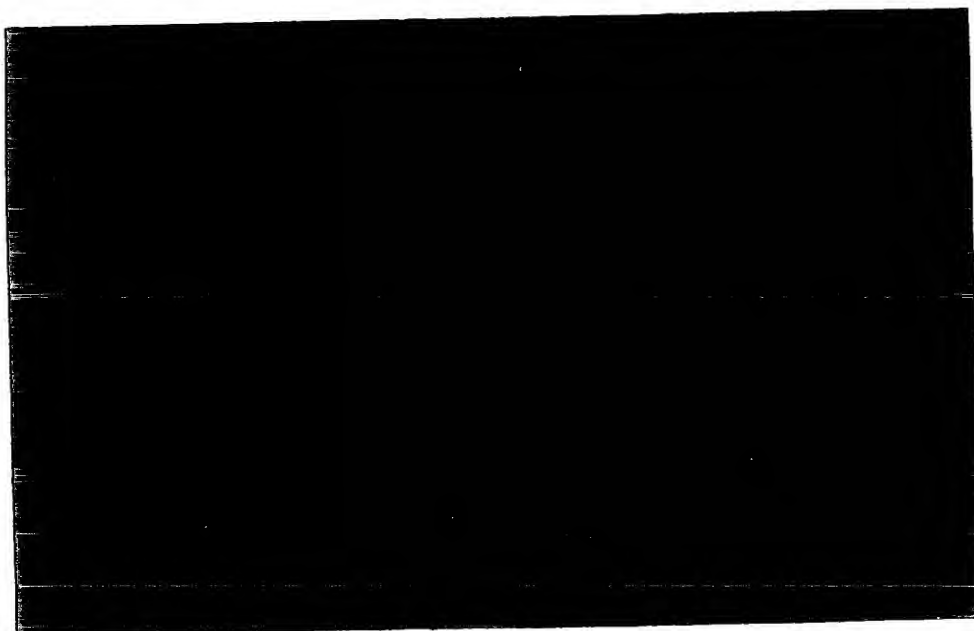LT unbound (Rb^E)   LT bound (Rb^H)

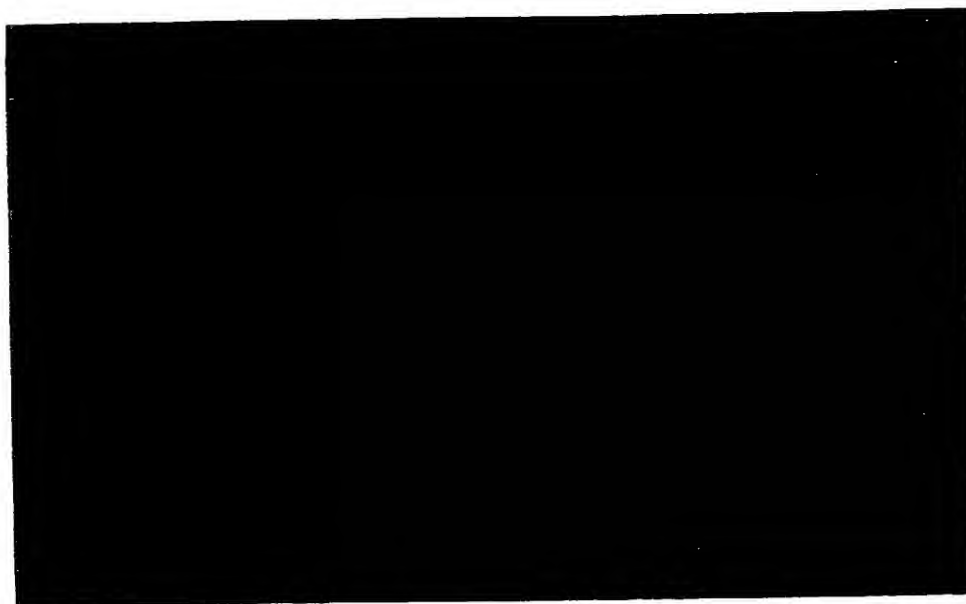FIG. 6

Untreated

FIG. 7A

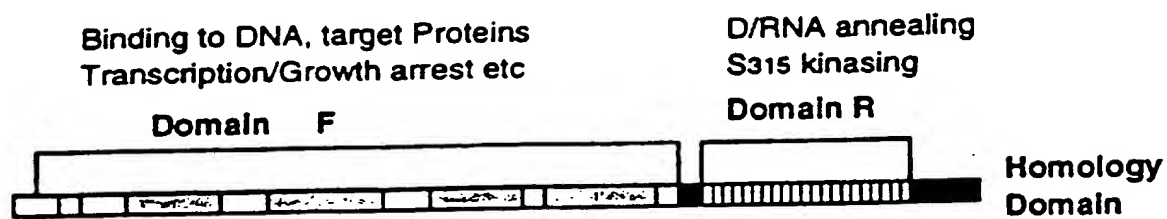TGFB treated
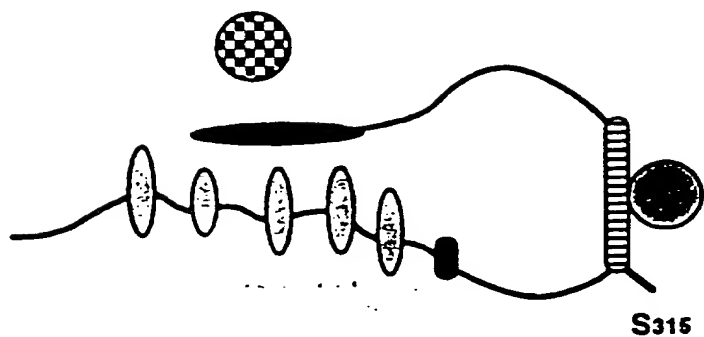for 48 hrs

FIG. 7B

7 / 13

FIG. 8A

FIG. 8B

FIG. 9A
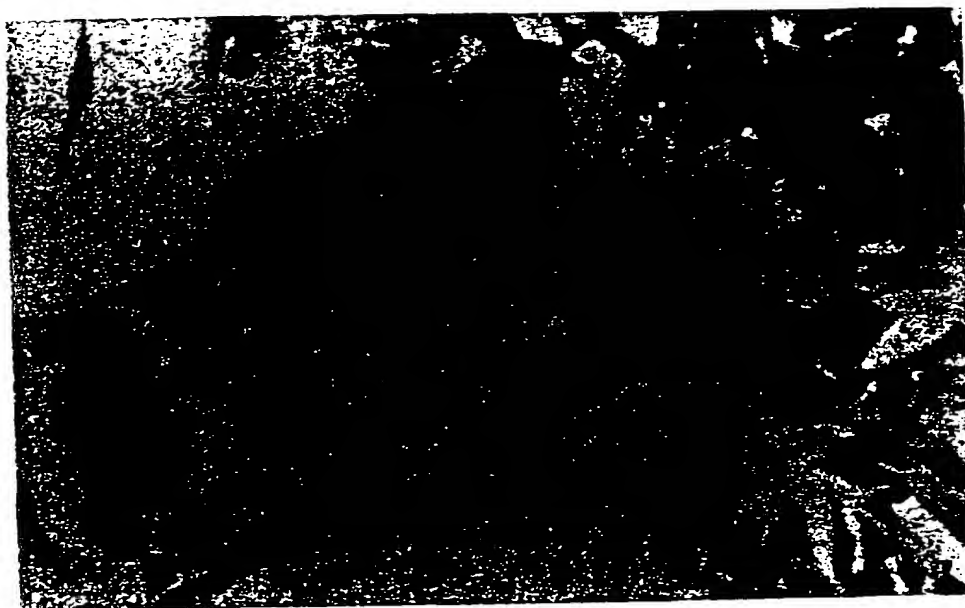
FIG. 9B

Binding to DNA, target Proteins
Transcription/Growth arrest etc

D/RNA annealing
S315 kinasing

Domain F

Domain R

Homology
Domain

Wild type (Domain F hidden, Domain R exposed)

S315

Mutant (Domain F exposed, Domain R hidden)

FIGURE 10

FIGURE 11

FIGURE 12

A.

	WT	P5	P3	P4	0	WT	P5	P3	P4
Lysate (ul)	5	5	5	5	5	1	1	1	. 1

FIGURE 13A

B.

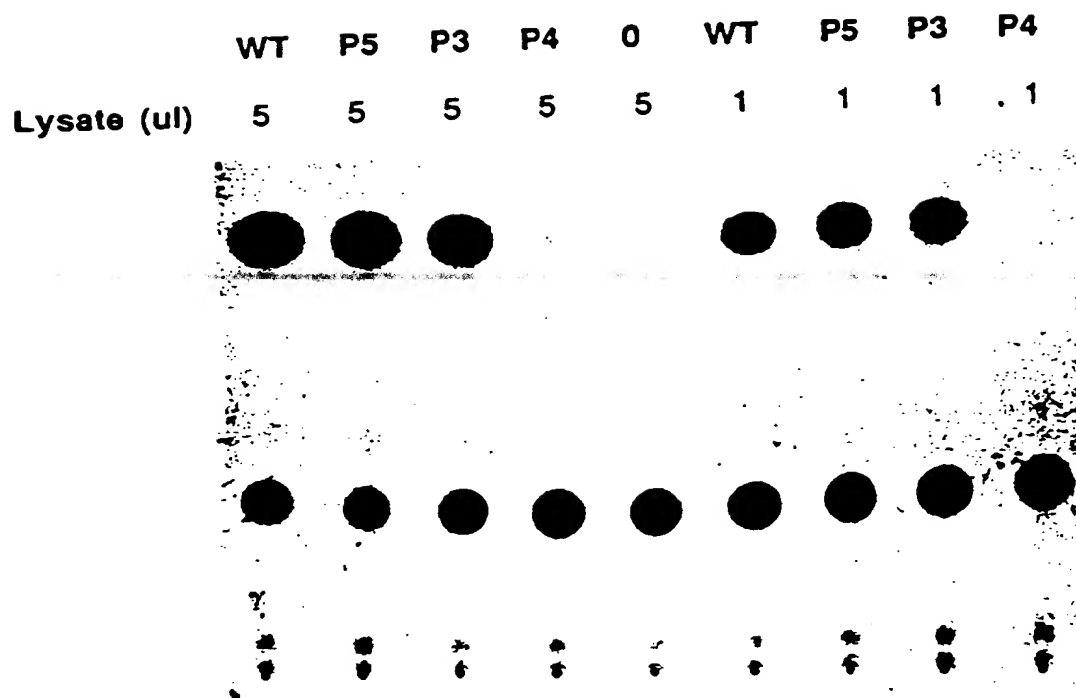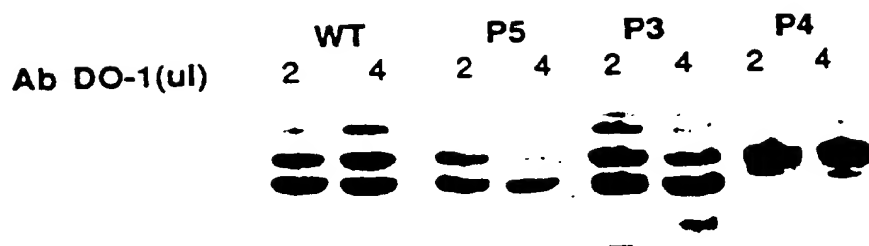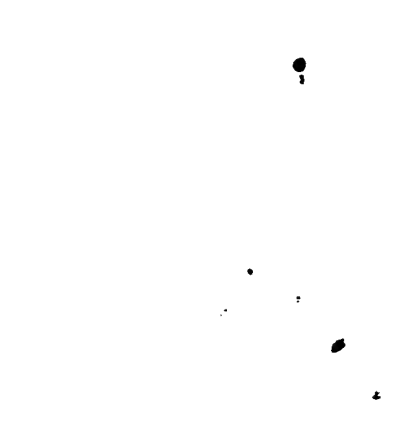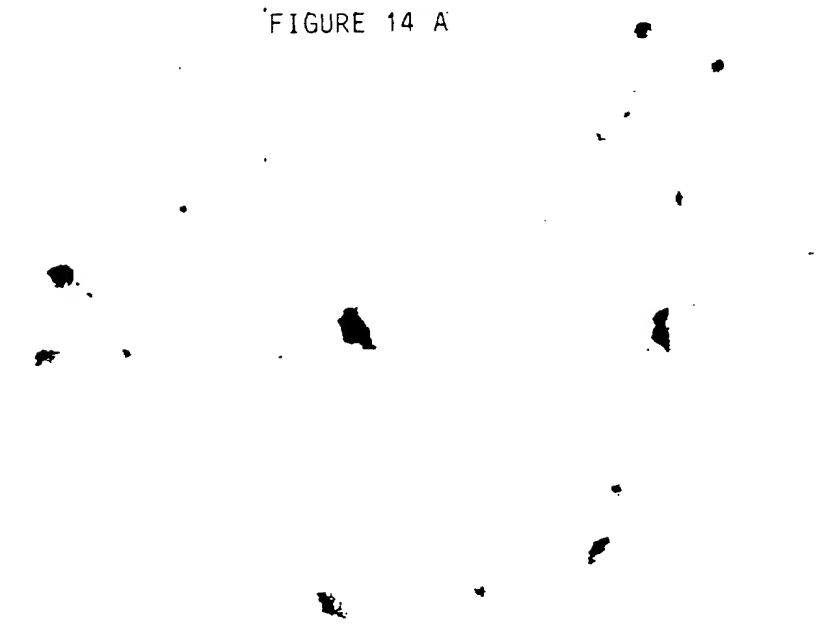| Ab DO-1(ul)	WT		P5		P3		P4	
	2	4	2	4	2	4	2	4
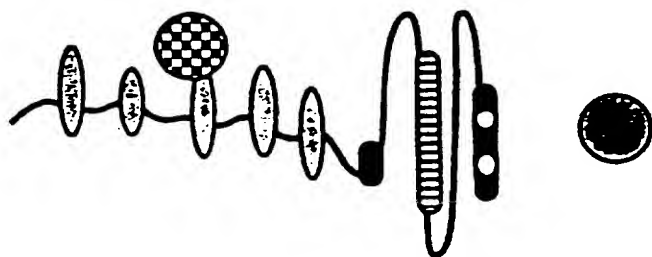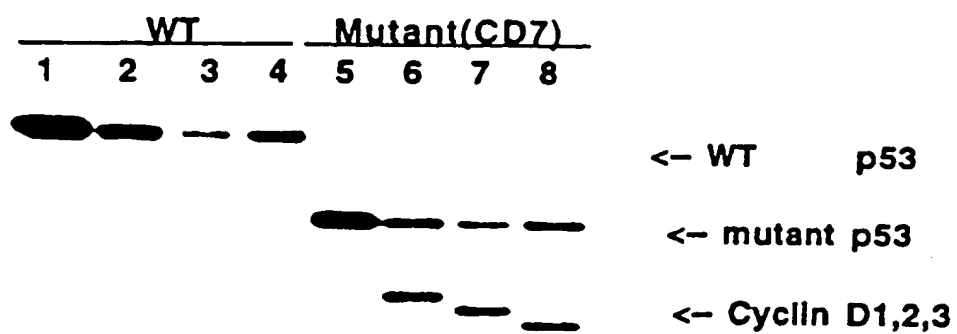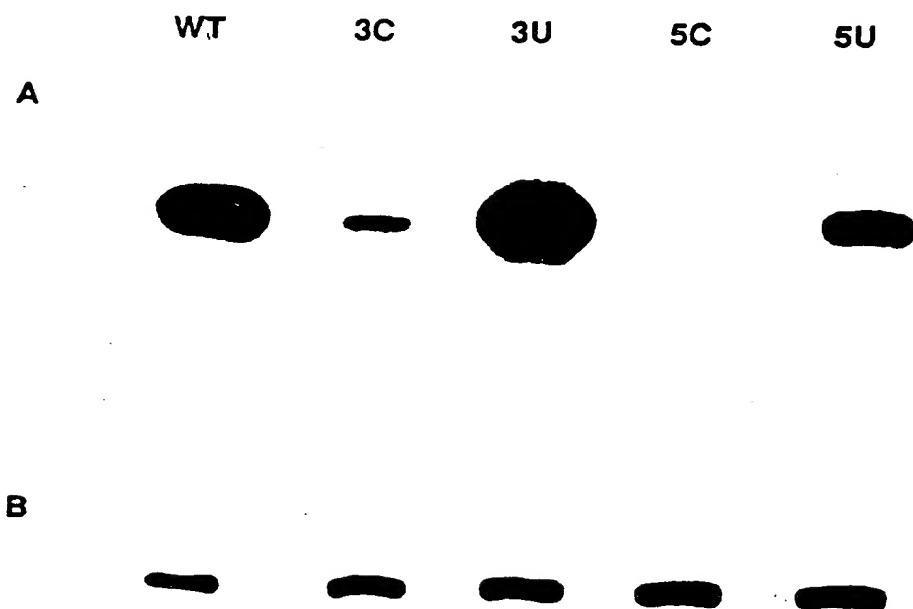
FIGURE 13B

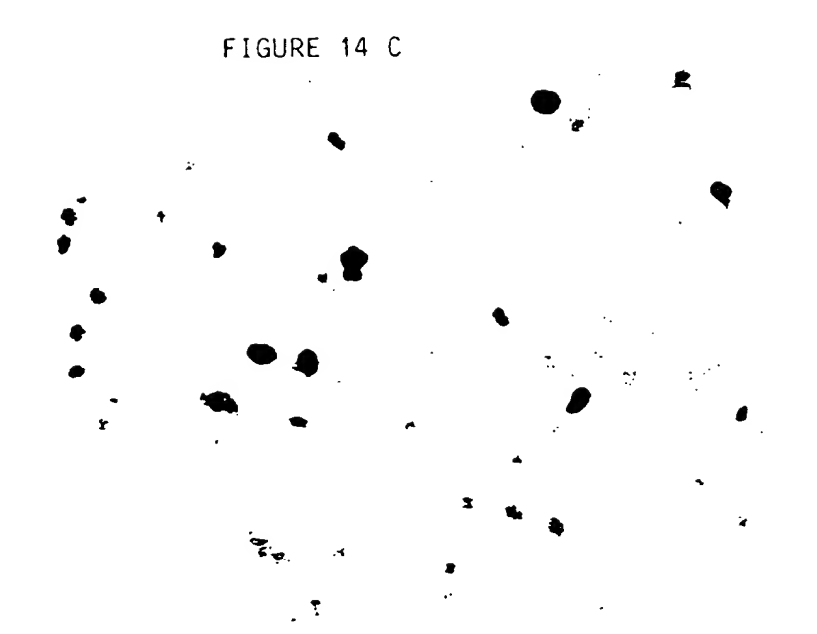FIGURE 14 A

FIGURE 14 B

FIGURE 14 C

FIGURE 14 D

FIGURE 14 E

FIGURE 14 F

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/17085

A.   CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C07H 21/04; A61K 31/70, 38/17; C12N 15/85; C07K 14/475
US CL   :514/44, 7, 12; 536/23.5; 530/352; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   514/44, 7, 12; 536/23.5; 530/352; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,942,123 (LEE et al.) 17 June 1990, column 10, lines 42 to 61, and column 11, lines 23-31.	1-44
Y	Science, Volume 265, issued 15 July 1994, Cho et al., "Crystal Structure of a p53 Tumor Suppressor-DNA Complex: Understanding Tumorigenic Mutations," pages 346-355, see entire document.	7-9, 11, 20-23, 30-33, 36, 37, 3-44
Y	Cancer, Volume 70, issued 1992, Friedmann, "Gene Therapy of Cancer Through Restoration of Tumor-Suppressor Functions?," pages 1810-1817, see pages 1813-1816.	1-41
Y	THE LANCET, Volume 339, issued 21 March 1992, Gutierrez et al., "Gene Therapy for Cancer," pages 715-721, see pages 719-720.	1-41

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 APRIL 1996	23 APR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized Officer CHARLES C. P. RORIES Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category[a]	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,049,386 (EPPSTEIN et al.) 17 September 1991, column 10, lines 12-20 and 43-62.	1-44
Y	Molecular and Cellular Biology, Volume 13, No. 6, issued June 1993, Hiebert, "Regions of the Retinoblastoma Gene Product Required for its Interaction with the E2F Transcription Factor Are Necessary for E2 Promoter Repression and pRb-Mediated Growth Suppression," pages 3384-3391, see entire document.	1-6, 10, 12-19, 24-29, 34, 35, 38, 40, 41
Y	Molecular and Cellular Biology, Volume 13, No. 8, issued August 1993, Zacksenhaus et al., "A Bipartite Nuclear Localization Signal in the Retinoblastoma Gene Product and its Importance for Biological Activity," pages 4588-4599, see entire document.	1-6, 10, 12-19, 24-29, 34, 35, 38, 40, 41

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark □ Protest ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, DERWENT WORLD PATENT INDEX, IG SUITE
search terms: retinoblastoma, rb, prb, p53, muta?, residue, amino acid, cdna, protein, administ?, suppress?, tumor, cancer, treat, therapy, therapeutic?, cationic(w)lipid, liposome

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I. Claims 1-6, 10, 12-19, 24-29, 34, 35, and 38, drawn to a mutant Rb gene and protein, and to methods for using said mutant Rb gene, classified in Class 514, subclass 44, for example.

II. Claims 7-9, 11, 20-23, 30-33, 36, 37, and 39, drawn to a mutant p53 gene and protein, and to methods for using said mutant p53 gene, classified in Class 514, subclass 44, for example.

III. Claims 40 and 41, drawn to a therapeutic method wherein mutant Rb and p53 genes are used together, classified in Class 514, subclass 44, for example.

IV. Claims 42-44, drawn to a therapeutic method wherein mutant p53 protein is administered, classified in Class 514, subclass 12, for example.

The mutant p53 gene and protein, and the methods for using said mutant p53 gene, are included together in Group II because the search of the methods along with the mutant p53 gene and protein is not considered to place an undue burden on the examiner.

The claims of Groups I, II, III, and IV lack unity of invention under 37 C.F.R. 1.475(d), as the claims of Groups II, III, and IV are each drawn to a product that is chemically different from the first-to-be claimed mutant Rb gene product of Group I, and/or are drawn to a method of using said product that is chemically different from the mutant Rb gene product of Group I. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2, so as to form a single inventive concept.

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PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07H 21/04, A61K 31/70, 38/17, C12N 15/85, C07K 14/475	**A1**	(11) International Publication Number: **WO 96/20207** (43) International Publication Date: 4 July 1996 (04.07.96)

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(71) Applicant (for all designated States except US): RESEARCH DEVELOPMENT FOUNDATION [US/US]; 402 North Division Street, Carson City, NV 89703 (US).

(72) Inventor; and
(75) Inventor/Applicant (for US only): FUNG, Yuen, Kai [US/US]; 2455 Ivanhoe Drive, Los Angeles, CA 90039 (US).

(74) Agent: WEILER, James, F.; Suite 1560, 1 Riverway, Houston, TX 77056 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: MUTANTS OF THE Rb AND p53 GENES AND USES THEREOF

(57) Abstract

The present invention provides mutants of the Rb and p53 genes and methods utilizing these mutants therapeutically. Along with mutated Rb genes and p53 genes, the present invention provides plasmids containing a mutated Rb gene or a p53 gene. In addition, the present invention provides cells transfected with the plasmids of the present invention. Moreover, the present invention provides for methods of treating a variety of pathophysiological cell proliferative diseases.

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MUTANTS OF THE Rb AND p53 GENES AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

5   The present invention relates generally to the field of regulation of cell growth and proliferative diseases.   More specifically, the present invention relates to mutants of the RB-1 and p53 genes and therapeutic uses of such mutants.

Description of the Related Art

10   The control of cell proliferation is a complex process which involves multiple interacting components.   Whether a cell grows or not depends on the balance of the expression of negatively-acting and positively-acting growth regulatory genes. Negatively-acting growth regulatory genes are those that, when

15 expressed in or provided to a cell, lead to suppression of cell growth.   Positively-acting growth regulatory genes are those which, when expressed in or provided to a cell, stimulate its proliferation.

Recently, several negatively acting growth regulatory

20 genes called tumor suppressor genes which have a negative effect on cell proliferation have been identified. These genes include, but are not limited to, the human retinoblastoma gene, RB-1, and the p53 gene. The absence or inactivation of some of these negative growth regulatory genes has been correlated with certain

25 types of cancer.

The human retinoblastoma gene, RB-1, is the prototype of this class of tumor suppressor genes in which the absence of both alleles of the gene in a cell or the inhibition of the expression of the gene or its gene product will lead to

30 neoplastic or abnormal cellular proliferation. At the molecular level, loss or inactivation of both alleles of RB-1 is involved in the clinical manifestation of tumors such as retinoblastoma and clinically r lated tumors, such as osteosarcomas, fibrosarcomas, soft tissue sarcomas and melanomas. In addition,

35 loss of the function of RB-1 has also been associated with other

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types of primary cancer such as primary small cell lung
carcinoma, bladder carcinoma, breast carcinomas, cervical
carcinomas and prostate carcinomas.

The Tumor Suppressor Genes Rb and p53 suppress the proliferation
of cancerous and noncancerous cells

The re-introduction of a wild-type cDNA of RB-1 or p53
have been shown to partially restore normal growth regulation as
the re-introduced genes induce growth arrest or retardation in
many different tumor cell types. The designation of the Rb gene
as a tumor suppression gene stemmed from the fact that
inactivation of an allele of the Rb gene is a predisposition to
the development of cancer. However, the growth suppression effect
of the Rb gene is not restricted to tumor cells. Normal cells
which have two copies can be growth arrested or retarded by the
introduction of extra copies of the Rb gene under certain growth
conditions. Likewise, the ability of a wild type p53 to suppress
the growth of noncancerous cells is well documented. Thus, the
step controlled by Rb and p53 may not directly affect the
tumorigenic phenotype but rather the steps that control the
growth of tumor and normal cells alike are affected.

Mechanisms of pathological proliferation of normal cells

There is a wide variety of pathological cell
proliferative conditions for which novel methods are needed to
provide therapeutic benefits. These pathological conditions may
occur in almost all cell types capable of abnormal cell
proliferation. Among the cell types which exhibit pathological
or abnormal growth are (1) fibroblasts, (2) vascular endothelial
cells, and (3) epithelial cells. It can be seen from the above
that methods are needed to treat local or disseminated
pathological conditions in all or almost all organ and tissue
systems of the individual.

For instance, in the eye alone, novel methods may be
utilized to treat such a wide variety of pathologic disease
states which are due to abnormal proliferation of normal, benign
or malignant cells or tissues including, but not limited to, the
following: fibroproliferative, vasoproliferative and/or
neoplastic diseases of the eye: retinopathy of prematurity,

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proliferative   vitreoretinopathy,   proliferative   diabetic
retinopathy, capillary hemangioma, choroidal neovascular nets,
subretinal neovascular nets, senile macular degeneration due to
subretinal,   neovascularization,   corneal   neovascularization,
5   macular pucker due to epiretinal membrane proliferation, adult
cataracts due to nuclear sclerosis, fibrous ingrowth following
trauma or surgery, optic nerve gliomas, angiomatosis retinae,
neovascular glaucoma, cavernous hemangioma, rubeosis iridis,
sickle cell proliferative retinopathy, epithelial downgrowth
10   after eye surgery or injury, after-cataract membrane, papilloma,
retinal   neovascularization   in   thalassemia,   subretinal
neovascularization due to pseudoxanthoma elasticum, and
neurofibromatosis type 1 and 11, retinoblastoma, uveal melanoma,
and pseudotumor of the orbit. Other benign cell proliferative
15   diseases for which the present invention is useful include, but
are not limited to, psoriasis, ichthyosis, papillomas, basal cell
carcinomas,   squamous   cell   carcinoma,   and   Stevens-Johnson
Syndrome.

It should be noted that in the case of normal cells,
20   there are already two normal alleles each of the Rb gene and the
P53 gene and yet these wild type proteins fail to prevent the
cells from proliferating when the cells were provided growth
factors, as for example in tissue culture growth conditions. The
uncontrolled proliferation of otherwise resting cells in
25   pathological conditions is also due to the exposure of the cells
to growth factors induced by the pathological conditions (see
below). For an example, uncontrolled proliferation of blood
vessels in the eye in thalassemia can lead to detachment of the
retina if the proliferation is not stopped. The introduction of
30   extra copies of the Rb gene or the P53 gene into such cells may
or may not be sufficient to suppress the cells from growing. In
fact, the introduction of exogenous Rb gene into many tumor cells
only retarded the growth rate of the cells instead of complete
arrest. The previously reported cell lines such as Saos-2 and
35   DU145 are good examples of this phenomenon. Many more copies of
the gene may need to be introduced and it is difficult to control
on the one hand the number of copies that need to be or could be

-4-

introduc d. On the other hand, growth factor exposure may lead
to inactivation of the expressed Rb proteins.

Regulation of the Activity of Rb Protein by Phosphorylation

         If the inactivation of growth suppressor genes is an
5   essential step in the removal of the constraint on cell growth,
     there must be mechanisms whereby the activities of these gene
     products are regulated so that a cell may proliferate in a
     controlled manner. It is conceivable that the expression of a
     given growth suppressor gene may be regulated quantitatively or
10   qualitatively. In the case of the Rb gene, the ratio of the
     steady state level of the protein to the cell volume is a
     constant throughout the cell cycle. This lack of variation of
     the Rb protein concentration suggests that there must be other
     mechanisms whereby a cell can regulate the activity of this
15   protein. There are several lines of evidence to show that the
     activity of the Rb protein (pRb) is regulated by its
     phosphorylation state. Various growth stimulatory or inhibitory
     factors exert their effects by perturbing the phosphorylation of
     growth suppressor gene products such as the Rb protein. Evidence
20   for a role of growth stimulatory factors in the induction of
     phosphorylation of the Rb proteins have come from the observation
     that the Rb proteins exist in the underphosphorylated forms in
     quiescent cells. In addition, when quiescent cells were
     stimulated to proliferate by exposure either to serum or to
25   growth factors such as EGF together with insulin and
     transferrin, the predominant form of the Rb protein was
     underphosphorylated in G1 but became hyperphosphorylated as the
     cells enter the G1/S boundary and the cells. These data suggest
     that the Rb protein is a target of the signal transduction
30   pathway induced by serum or growth factors. Finally, senescent
     human fibroblast cells incapable of responding to the
     proliferation stimulatory effects of growth factors also failed
     to phosphorylate the Rb protein.

         There are also evidence for a role of growth inhibitory
35   factors in the downregulation of phosphorylation of the Rb
     proteins. When actively growing leukemia or neuroblastoma cells
     were treated with retinoic acid or vitamin D3, DMSO other

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differentiation inducing reagents, the Rb proteins were found to
be underphosphorylated prior to the on set of cell growth arrest
in the early G1 phase and induced to differentiation.   In
addition, the treatment of lung epithelial cells with the
5   paracrine growth inhibitory polypeptides-transforming growth
factor Beta 1 (TGFβ-1) led to the downregulation of
phosphorylation of the Rb protein and concomitant arrest of c ll
growth in late G1.   Taken together, these data suggest the
underphosphorylated form of the Rb protein actively suppressed
10   cells from traversing the G1/S boundary and that a kinase (S),
activated in G1, can inactivate the Rb protein so that the cells
may move into the S phase.

        While the permanent removal of the Rb gene, by deletion
or mutation, in tumor cells allowed growth, removal of the
15   constraint on growth in normal cells having normal expression of
the Rb gene is achieved by cell cycle dependent post-
translational modification of its gene product, i.e., the Rb
protein (pRb).   The Rb protein exists in multi-phosphorylated
forms and evidence suggests that the underphosphorylated form is
20   the active form.   For example, the SV40 large T antigen
preferentially binds to the underphosphorylated form of the Rb
protein.   Phosphorylation of the Rb protein releases it from the
SV40 large T antigen and only the underphosphorylated forms of
pRb binds to and inhibits transcription from the E2 promotor.
25   Thus, the underphosphorylated form of the Rb protein actively
suppresses cell growth, whereas cell proliferation is associated
with hyperphosphorylation of the Rb protein.   Various growth
stimulatory or inhibitory factors may exert their effects by
perturbing the phosphorylation of the Rb protein.   Therefore,
30   although a normal cell may be expressing the Rb proteins, it can
be induced to grow when exposed to appropriate growth factors
such as may be found in various growth conditions, including
pathological conditions.   Under certain pathological conditions,
an otherwise normal cell may be induced to proliferate
35   undesirably.   Proliferation of normally quiescent fibroblasts in
the eyes in diabetic retinopathy is an example of such unwanted
induction of cell growth.   Left untreated, the proliferating

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fibroblasts will eventually attach to the retina and lead to its rapid detachment.

Activation of the p53 protein

Similar to the Rb gene, inactivation or mutation of the
5   p53 gene has been frequently observed in tumor and nontumorous cell lines. Wild type p53 protein is also negatively regulated when it binds to viral antigens such as the SV40 large T. The p53 protein functions as a transcription factor and binds to specific DNA sequences. The ability of the wildtype p53 protein
10   to bind DNA is critical for it to function properly. In mutant p53, the ability of the protein to bind to specific DNA target sequences or to activate transcription is lost, suggesting that these activities are important for the suppressive function of the protein. However, it has also been recently shown that
15   sequence specific DNA binding activity of the p53 protein is cryptic. Newly synthesized p53 protein is inactive in that it cannot bind to its specific DNA sequence unless the protein is modified. One of the sites for modification is amino acid residues 365-393 at the C-terminus. This region is the site for
20   the attachment of an RNA moiety as well as for the binding to the bacterial heat shock proteins dnaK. Binding of an antibody, Pab421, to the 365-389 amino acid residues activates the protein by altering its conformation so that it can bind to its specific DNA sequence.

25   Difference in the mode of function of pRb and p53

Although both Rb and p53 are regarded as tumor suppressor genes, their modes of action are very different. Previously, evidence was provided that these two genes may work on different pathways. Mice null for the Rb gene is
30   embryonically lethal while mice null for p53 develop normally although they are more susceptible to cancer development. At the cellular level, cancer cells that are devoid of p53 can be growth arrested or retarded by the overexpression of the Rb gene even in the absence of exogenous p53. On the other hand, cells devoid
35   of Rb are susceptible to the growth suppression effect of the p53 gene. At the molecular level, p53 and pRb interacts with

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different protein targets and DNA sequences (directly or indirectly).

The use of the Rb and p53 gene in therapy

While these two proteins are different in their modes
5   of action, they are similar in one aspect, i.e., overexpression
of the active forms of the two proteins under experimental
conditions lead to the suppression or retardation of growth of
cells. Knowing that cell growth is controlled by the expression
of suppressor genes such as pRb and p53 and that their proteins
10   need to be in the active conformations in order to function, it
is desirable to have active forms of these genes for gene
therapy. In the case of pRb, the newly synthesized proteins are
underphosphorylated and are active. Inactivation may occur as the
cells are exposed to growth factors. It would be desirable to
15   have mutant forms of pRb that would remain in the active form and
are resistant to inactivation by phosphorylation. In the case
of p53, the newly synthesized protein is inactive in that it
cannot bind DNA unless modified. In gene therapy, it would be
highly desirable to have mutant forms of the p53 gene, the
20   protein of which is already active even without further
modification. Finally, since the two proteins have different
modes of action, it would be desirable to use a combination of
both the Rb and the p53 genes in therapy such that the mutations
or growth conditions that renders a cell resistant to the growth
25   suppression effect of one gene may be susceptible to the other.

The prior art remains deficient in the lack of a tumor
suppressor gene mutated so that the gene product (protein) is
permanently active and can function even when the cell is exposed
to growth factors. More specifically, the prior art is deficient
30   in the lack of effective and functional mutated forms of the p53
and Rb genes. The prior art also remains deficient in the
simultaneous application of the wildtype and/or the mutant forms
of the Rb and the p53 genes in cancerous and noncancerous cell
proliferative diseases.

35   SUMMARY OF THE INVENTION
In one aspect, the present invention provides a
generalized approach to the treatment of inappropriate or

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pathological cell growth such as exists in non-cancerous cell proliferative diseases as well as cancerous cell proliferative diseases. More specifically, the present invention provides methods of treating pathophysiological cell proliferative
5   diseases by administering mutated growth suppressor gene and /or gene products, i.e., mutated proteins.

In one embodiment of the present invention, there is provided an isolated DNA molecule, wherein said molecule is a synthetic mutated Rb gene encoding a mutated functionally active
10   retinoblastoma protein.

In another embodiment of the present invention, there is provided an isolated DNA molecule, wherein said molecule is a synthetic mutated p53 gene encoding a mutated functionally active p53 protein.

15   In another embodiment of the present invention, there is provided a novel plasmid containing a functionally active mutant of the retinoblastoma or p53 gene.

In yet another embodiment of the present invention, there is provided a method of the consecutive or simultaneous
20   administration of the wild type and/or the active mutants of both the Rb and the p53 genes for therapeutic purposes.

In other embodiments of the present invention, there are provided methods for the treatment of pathological cell proliferative diseases comprising administration concurrently or
25   consecutively to a non-cancerous proliferating cell a mutated Rb gene and a mutated P53 gene.

Additionally provided is a method of treating malignant cell diseases in individuals comprising administration concurrently or consecutively into a proliferating cancer cell
30   of a mutated Rb gene and a mutated P53 gene.

In yet another embodiment of the present invention, there is provided a method of inducing apoptosis, comprising the step of administering the mutant p53 protein of the present invention to a cell.

35   In yet another embodiment of the present invention, there is provided a method of treating a tumor cell having a

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mutation of a cyclin protein, comprising the step of administering the protein of claim 39 to a cell.

Other and further aspects, features, and advantages of the present invention will be apparent from the following
5   description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the discovery of the homology region between pRb and p53 by immunoprecipitation of cellular proteins
10   using polyclonal anti-Rb antibodies RB1-Ab #18 and #20.

Figure 2 shows the confirmation of the homology region between pRb and p53 immunoprecipitation of cellular protein using polyclonal anti-Rb antibodies RB1-Ab #18 and #20.

Figure 3 shows the ability of the P1, P3, P5 mutant p53
15   proteins to bind specific DNA sequences in the absence of Pab421.

Figure 4 shows the two dimensional mapping analysis of the phosphorylation pattern of mutant Rb proteins as compared to those of the wildtype, (A) in vivo labelled proteins (B) in vitro labelled proteins.

20   Figure 5 shows the binding of the retinoblastoma protein to the SV40 Large T antigen and the dissociation of the complex by phosphorylation.

Figure 6 shows the two dimensional mapping analysis of two different forms of phosphorylated retinoblastoma protein
25   (those that are bound by SV40 large T and those that are not).

Figure 7 shows the two dimensional mapping analysis of the change in the phosphorylation pattern of the retinoblastoma protein under different growth conditions.

Figure 8 shows the ability of the m89 Rb mutant to
30   suppress the growth of the normal cell WS1.

Figure 9 shows the ability of the m89 Rb mutant to suppress the growth of the bladder tumor cell line TCCSUP.

Figure 10 shows a schematic diagram of both the wild type p53 protein showing the domain F and domain R and the mutant
35   p53 protein showing the domain F and domain R.

Figure 11 shows that the mutant p53 protein is able to bind specific DNA sequences.

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Figure 12 shows that the homology regions control the exposure of the C-terminus of p53 so that specific sites are not phosphorylated.

Figure 13 shows that the homology region mutants of p53
5   are transcriptionally active *in vivo*. Figure 13A shows that p53 mutants of the p3 or p5 homology region at the conserv d aminoacids is active in promoting transcriptional activation. Figure 13B demonstrates that equal amount of proteins were used for all the p53 constructs.

10   Figure 14 shows that the synthetic p53 mutant induces apoptosis in the lung carcinoma cell line, H358. Figure 14A, wild type p53 without cisplatin; Figure 14B, wild type p53 + cisplatin; Figure 14C, P4 p53 mutant without cisplatin; Figure 14D, P4 p53 mutant + cisplatin; Figure 14E, P5 p53 mutant without
15 cisplatin; Figure 14F, P5 p53 mutant + cisplatin.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "functional expression of the gene" is meant to include the suppression of transcription of the gene, the
20 degradation of the gene transcript (pre-message RNA), the inhibition of splicing, the destruction of the message RNA, the prevention of translation of the message RNA, the prevention of the post-translational modifications of the protein, the destruction of the protein, or the inhibition of the normal
25 function of the protein.

The term "transfected plasmid" is meant to include the bacterial plasmid which contains a mutated retinoblastoma or p53 gene to be carried (transfected) into the cell of choice.

The term "gene therapy" is meant to include the
30 insertion of part or all of a gene, a DNA construct, RNA, or gene product into a cell, group of cells, tissue, pathologic lesion, organ or organism for the purpose of modulating gene expression, and/or function of the gene product.

The term "prophylactic gene therapy" is meant to
35 include genes which may be used for partial or total inhibition or prevention of disease and the spread of disease and also is meant to include genes which may be used to supplement or replace

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absent or defective negative growth in cell, tissues or germlines.

The term "cell proliferative disease" is meant to include any human or animal disease or disorder, affecting any
5   one or any combination of organs, cavities or body parts, which is characterized by single or multiple local abnormal proliferations of cells, groups of cells or tissue(s), whether benign or malignant.

The term "prokaryote" is meant to include all bacteria
10   which can be transformed with the DNA for the expression of the recombinant molecules of the present invention.

The term "eukaryote" is meant to include all yeasts, fungi, animal and plant cells which can be transformed with the DNA for the expression of the recombinant molecules of .the
15   present invention.

The DNA for DNA constructs of the present invention can be synthetic or may be derived from any mammalian species. All that is required is that the genetic sequence for the Rb or p53 genes be functionally expressed in the prokaryotic or eukaryotic
20   organism. Preferred is synthetic DNA.

A recombinant DNA molecule coding for the DNA constructs of the present invention can be used to transform a host using any of the techniques commonly known to those of ordinary skill in the art.

25   Methods for preparing fused, operably linked genes and expressing them in bacteria are known and are shown, for example, in U.S. Patent No. 4,366,246, herein incorporated by reference. The genetic constructs and methods described therein can be utilized for construction of the DNA constructs of the present
30   invention and transfection in prokaryotic or eukaryotic hosts.

Prokaryotic hosts may include Gram negative as well as Gram positive bacteria, such as *E. coli*, *S. tymphimurium*, *Serratia marcescens* and *Bacillus subtilis*. Eukaryotic hosts may include yeasts such as *Pichia pastoris* or mammalian cells.

35   In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted DNA fragment are used in connection with the host. The

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expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells. The transformed hosts can be fermented and cultured according to
5   means known in the art to achieve optimal cell growth.

Examples of promoters which can be used in the invention include, but are not limited to: human β-actin promotor, metallothionin promotor, SV40 origin of replication, MMTV LTR promotor and MuLV LTR promotor. Examples of some of the
10   plasmids or bacteriophage which can be used in the invention are listed in Maniatis et al., Molecular Cloning, Cold Spring Harbor Laboratories, 1982, and others are known to those of skill in the art and can be easily ascertained.

A gene is a DNA sequence which encodes through its
15   template or messenger RNA a sequence of amino acids characteristic of a specific peptide. The term cDNA includes genes from which the intervening sequences have been removed. The term "recombinant DNA" (rDNA) is meant to include a molecule that has been recombined by splicing cDNA or genomic DNA
20   sequences in vitro.

A cloning vehicle is a plasmid or phage DNA or other DNA sequence which is able to replicate in a host cell which is characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a
25   determinable fashion without loss of an essential biological function of the DNA, and which contains a marker suitable for use in the identification of transformed cells.   Markers, for example, are tetracycline resistance or ampicillin resistance. The word "vector" is sometimes used for a cloning vehicle.

30   An expression vehicle is a vehicle similar to a cloning vehicle but which is capable of expressing a given structural gene in a host, normally under control of certain control sequences.

The term "individual" is meant to include animals and
35   humans.

The term "biologically inhibiting" or "inhibition" of the growth of proliferating cells is meant to include partial or

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total growth inhibition and also is meant to include decreases in the rate of proliferation or growth of the cells. The biologically inhibitory dose of the mutants of the present invention may be determined by assessing the effects of the test

5   element on target malignant or abnormally proliferating cell growth in tissue culture, tumor growth in animals and cell culture or any other method known to those of ordinary skill in the art.

As used herein, the term "conserved homology region"

10 refers to that amino acid region that is homologous between Rb and p53 as illustrated in Table I.

Administration of the proteins of the present invention may be by topical, intraocular, parenteral, oral, intranasal, intravenous, intramuscular, subcutaneous, or any other suitable

15 means. The preferred method of administration for treatment of ocular diseases is intraocular or periocular injection. The preferred method of administration for treatment of skin cell proliferative diseases is by topical application or subcutaneous injection.

20   In another embodiment, the DNA constructs of the present invention may be delivered to the focal proliferative disease directly. In the case of ocular proliferative disease, the DNA constructs of the present invention may be directly injected into the eye. The DNA constructs of the present

25 invention may be delivered directly to focal disease sites in internal organs, body cavities and the like by use of imaging devices used to guide the injecting needle directly to the disease site. The DNA constructs of the present invention may also be administered to disease sites at the time of surgical

30 intervention.

The DNA dosage administered is dependent upon the age, clinical stage and extent of the disease or genetic pr disposition of the individual, location, weight, kind of concurrent treatment, if any, and nature of the pathological or

35 malignant condition. The effective delivery system useful in the method of the present invention may be employed in such forms as capsules, tablets, liquid solutions, suspensions, or elixirs, for

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oral administration, or sterile liquid forms such as solutions,
suspensions or emulsions. Any inert carrier is preferably used,
such as saline, or phosphate-buffered saline, or any such carrier
in which the compounds used in the method of the present
5   invention have suitable solubility properties.

Preferably, for intraocular delivery and for delivery
to other localized disease sites, delivery systems useful in the
method of the present invention may be employed in such sterile
liquid forms such as solutions, suspensions or emulsions. For
10   topical use it may be employed in such forms as ointments, creams
or sprays. Any inert carrier is preferably used, such as saline,
or phosphate-buffered saline, or any such carrier in which the
compounds used in the method of the present invention have
suitable solubility properties.

15   For local administration to cells, administration may
be by any method known to those of skill in the art including,
but not limited to, transfection, electroporation, microinjection
of cells, or in vehicles such as liposomes, lipofectin or as
naked DNA or RNA. The DNA of the present invention may be
20   delivered by known gene delivery systems such as, but not limited
to, retroviral vectors (Gilboa (1982) J. Virology 44:845; Hocke
(1986) Nature 320:275; Wilson et al., Proc Natl Acad Sci USA
85:3014), vaccinia virus system (Chakrabarty et al., (1985) Mol.
Cell Biol. 5:3403) or other efficient DNA delivery systems (Yates
25   et al., (1985) Nature 313:812) known to those of skill in the
art. These references are exemplary only. In order to
specifically deliver or transfect cells which are abnormally
proliferating and spare non-dividing cells, it is preferable to
utilize a retrovirus delivery system known to those of skill in
30   the art. Since host DNA replication is required for retroviral
DNA to integrate and the retrovirus will be unable to self
replicate due to lack of the retrovirus genes needed for its life
cycle, utilizing such a retroviral delivery system the present
invention will target said anti-proliferative elements to
35   abnormally proliferating cells and will spare non-dividing normal
cells from any intervention.

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        The mutant proteins of the present invention may be
administered in any biologically effective carrier.   Th
biologically effective carriers may include retroviruses,
liposomes, and any other transfection mechanism capable of
5   introducing foreign proteins into the genome of the cell. Such
transfection mechanisms are known to those of skill in the art.
The carrier may also include any agent or solvent with which the
constructs of the present invention are compatible and which are
non-toxic to the individuals or cells treated at the amounts
10   administered.
     Pathological cell proliferative conditions to be treated
        There is a wide variety of pathological cancerous and
noncancerous cell proliferative conditions for which the method
of the present invention will provide therapeutic benefits.
15   These pathological conditions may occur in almost all cell types
capable of abnormal cell proliferation.   Among the cell types
which exhibit pathological or abnormal growth are (1)
fibroblasts, (2) vascular endothelial cells and (3) epithelial
cells.   It can be seen from the above that the method of the
20   present invention is useful in treating local or disseminated
pathological conditions in all or almost all organ and tissue
systems of the individual.
        For instance, in the eye alone, the method of the
present invention may be utilized to treat such a wide variety
25   of pathologic disease states caused by abnormal proliferation of
normal (benign) or malignant cells or tissues including, but not
limited to, the following fibroproliferative, vasoproliferative
and/or neoplastic diseases of the eye: retinopathy of
prematurity, proliferative vitreoretinopathy, proliferative
30   diabetic retinopathy, capillary hemangioma, choroidal neovascular
nets, subretinal neovascular nets, senile macular degeneration
due to subretinal, neovascularization, corneal
neovascularization, macular pucker due to epiretinal membrane
proliferation, adult cataracts due to nuclear sclerosis, fibrous
35   ingrowth following trauma or surgery, optic nerve gliomas,
angiomatosis retinae, neovascular glaucoma, cavernous hemangioma,
rubeosis iridis, sickle cell proliferative retinopathy,

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epithelial downgrowth after eye surgery or injury, after-cataract
membrane, papilloma, retinal neovascularization in thalassemia,
subretinal neovascularization due to pseudoxanthoma elasticum,
and neurofibromatosis type 1 and 11, retinoblastoma, uveal
5   melanoma and pseudotumor of the orbit.   Other benign cell
proliferative diseases for which the present invention is useful
include, but are not limited to, psoriasis, ichthyosis,
papillomas, basal cell carcinomas, squamous cell carcinoma and
Stevens-Johnson Syndrome.
10   In general, the present invention is applicable to all
forms of cancer including but not limited to cancers in which the
inactivation of the Rb gene and/or the p53 gene have been
implicated such as osteosarcoma, soft tissue sarcomas, melanomas.
fibrosarcoma, retinoblastoma, carcinoma of the breast, bladder,
15   cervix, lung, colon, ovary, kidney, pancreas, and prostate.
Furthermore, the novel mutant p53 proteins may be used to induce
apoptosis in various cells. Thus, the present invention also is
directed to a method of inducing apoptosis comprising the step
of administering a novel mutant p53 protein of the present
20   invention.
            In addition, the present invention provides a method
of treating a tumor cell having a mutation of a cyclin protein,
comprising the step of administering a novel mutant p53 protein
of the present invention to a cell. Representative mutations,
25   e.g., translocations, amplifications of cyclin D1, cyclin D2 or
cyclin D3 may be treated with the novel mutant p53 proteins of
the present invention.

EXAMPLE 1

A conformation determining domain conserved between pRb and p53

30   The present invention discloses a conserved homologous
structural domain that controls the conformations of the two
tumor suppressor gene products, Rb and p53, thus allowing the
creation of mutants of these genes and methods for their use.
Thus, the present invention provides an isolated DNA molecule,
35   wherein said molecule is a mutated growth suppressor gene. In
on pref rred embodiment of the present invention, the mutated
growth suppressor gene is a mutated Rb gene.   In another

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preferred embodiment of the present invention, the mutated growth
suppressor gene is a mutated P53 gene.

In an attempt to understand the interaction between pRb
and its cellular targets, antibodies to the various hydrophilic
5   regions of the Rb protein were raised. Since different regions
of the Rb protein may be interacting with different proteins, it
was necessary to raise antibodies to many different regions such
that some of the antibodies would bring down the protein complex
between the Rb protein and its targets without interfering with
10 their interaction. Of a panel of polyclonal anti-Rb antibodies
raised against synthetic peptides with sequences corresponding
to various hydrophilic domains, two of them (Rb1-Ab 18 and Rb1-
Ab20) consistently bring down, in addition to the Rb protein, a
protein of molecular mass 53 kD (Figure 1A, lanes 1, 2, 8 and 9).
15 These two antibodies turned out to be raised against the same
synthetic peptide, p5. The appearance of the 53 kD protein is
specific for antisera raised against p5. Antisera against other
regions, for example Rb1-Ab B (Figure 1, lanes 4-5) and Rb1-Ab
C (Figure 1, lanes 6-7) did not immunoprecipitate the 53 kD
20 protein although they can still recognize the Rb protein.
Because of the similarity in the molecular mass of this protein
and that of p53, it appears that this was the p53 protein itself.
Immunoprecipitation of the cell lines with Pab122, a monoclonal
antibody against p53 showed that the p53 indeed has the same
25 exact molecular mass as that of the 53 kD protein (lane 10).
Furthermore, if p53 was first removed from the cell lysate with
Pab 122, neither Rb1-Ab 18, or Rb1-Ab20 can immunoprecipitate the
53 kD protein, although they still can precipitate the Rb protein
(lane 11). To further verify that the 53 kD protein was indeed
30 p53, immunoprecipitation was done on several cell lines which
expressed no endogenenous p53 and/or Rb protein. In all cases
(lanes 12-20) the antibody indeed can recognize the p53 protein
in accordance with the status of the presence or absence of the
p53   gene   in   these   cell   lines.   Furthermore,   the
35 immunoprecipitation of p53 is independent of the presence or
absence of the Rb protein, suggesting that a complex was not

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formed and that the p53 protein was recognized by the antibodies directly.

To definitively show that the P53 protein shares a common antigenic domain with the Rb protein, a search of the amino acid sequence of the p53 protein corresponding to the P5 region of the Rb protein was initiated. If the 3-dimensional structural information was retained in these two proteins and in the P5 peptide, there may be a corresponding region on the P53 protein. Furthermore, a peptide with this conserved antigenic domain should block the immunoprecipitation of P53 by RB1-Ab 18 or RB1-Ab 20. Immunoprecipitation of Rb and p53 proteins by Rb1-Ab18 (Figure 2A) were performed in the presence of various amounts of these peptides. One such p53 peptide, F19, completely blocked the immunoprecipitation of p53 by Rb1-Ab18 at the lowest concentration (10 ug/ml) tested (Figure 2A, lanes 12-14). This was also the same concentration of the Rb peptide p5 used to block the precipitation (lanes 1 and 2). In contrast, all other p53 peptides failed to block the immunoprecipitation of p53 by Rb1-Ab18, even at 50 to 100 times the concentration used (lanes 3-11).

The p5 and F19 region are not the only regions that show structural homology between the Rb and p53 proteins. At least two other regions were identified by visual and computer search. For comparison, the homology regions of Rb and p53 are shown in Table I.

TABLE I

Comparison of the homology regions between Rb and p53

(P1 region) (67% homology)

| Rb --- | EINSALVLK | (184-192) |
| P53 -- | ELNEALELK | (343-351) |

(P3 region) (67% homology)

| Rb --- | RRGQNRSAR | (245-262) |
| P53 -- | KKGQSTSRH | (372-380) |

(P5 region) (62% homology)

| Rb --- | KKLRFDIEG--SD | (873-886) |
| P53 -- | KKLMFKTEGPDSD | (381-393) |

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Although these regions may appear to be short, those
amino acids that are homologous between pRb and p53 also are the
most evolutionarily conserved ones in p53 (there is no data
available for the sequence of Rb in different species). This
5   high degree of homology between the two growth suppressor
proteins suggest that these regions may have important functions.
A schematic drawing of the positions of the homology regions on
pRb and p53 is shown in Figure 2B. In p53, all three regions are
clustered in the C-terminus of the protein and in pRb regions 1
10   and 3 are clustered in the N-terminal half while the P5 region
is at the C-terminal half. However, in both p53 and pRb these
three regions are all located outside of the domains known to be
important for the suppression function of the proteins. If these
regions are not responsible for the physical interaction with
15   their suppression targets, it may be that they are responsible
for the regulation of the interaction process. Just as it is
important to have active Rb and p53 proteins to suppress
unwarranted cell growth, it is equally important to have a means
to down-regulate their activities so that a cell may proliferate.
20   The presence in these two growth suppressor gene products of the
conserved domains may ensure their coordinated regulation.
Indeed, as is shown below, these homology regions appear to form
a domain which determines the conformations of the two proteins.
Specifically, disruption of this domain by mutation at the
25   conserved amino acids residues (underlined in Table I above) of
any one of these three regions conferred upon the two proteins
an active conformation.   In contrast, mutations at the
unconserved amino acids in these regions have no effect.

EXAMPLE 2

30   Figure 10 shows a schematic representation of the
conformations of the wildtype and the mutant p53. Two domains
are described, domain F (function) and domain R (regulation).
Domain F is the region of p53 containing the five domains
conserved in different vertebrate species. Naturally occurring
35   mutation at one of these five domains lead to the inactivation
of the wildtype function of p53. The present inv ntion shows
that domain F is exposed in the Rb/p53 mutant but not in the

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wildtype with important functional consequence. Domain R
represents the C-terminus motif that is just outside of the
domain needed for the binding of p53 to specific DNA sequences.
This domain contains the Rb/p53 homology domain p1, p3, p5.
5   Domain R is hidden in the Rb/p53 mutant but not in the wildtype
with important functional consequences as detailed by the present
invention. Figure 10 shows a schematic diagram of both the wild
type p53 protein showing the domain F and domain R and the mutant
p53 protein showing the domain F and domain R. In Figure 3, the
10   synthetic mutant p53 was shown to bind to specific DNA
sequences, suggesting that the synthetic mutant p53 is assuming
a conformation different from that of the wildtype. Thus, the
DNA binding domain of the synthetic mutant p53 may also be able
to bind to other proteins that can to bind to domain F.

15   EXAMPLE 3
P1, P3, P5 regions control p53 conformation and DNA binding
ability

        The ability of p53 to activate transcription is tightly
related to its ability to bind DNA. Mutant p53 found in cancer
20   cell lines are unable to bind to DNA and unable to activate
transcription. The P53 protein is capable of binding to specific
DNA sequence in the presence of the antibody Pab421. A close
inspection of the epitope of the Pab421 revealed that it overlaps
with the P3 region. The epitope recognized by Pab421 (see Figure
25   3) is the last four amino acid residues in the P3 region. It
appears that blocking this entire P3 region with an antibody
(Pab421) sufficiently changed the conformation of the protein
allowing the p53 protein to bind with a specific DNA sequence.
The DNA binding assay therefore provided a convenient means of
30   testing the role of the P3 and the other homologous regions P1,
and P5 in the control of the conformation of the P53 protein.
As is shown in Figure 3, while the wild type p53 was unable to
bind to its specific DNA sequence (lane 1), it does bind in the
presence of the antibody Pab421 (lane 2). When the conserved
35   amino acids of either the P1 (lane 3), P3 (lanes 5 and 7) or P5
(lane 9) regions of p53 was mutated, the resulting p53 mutant
proteins were able to bind to DNA even in the absence of Pab421.

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The resulting complex was supershifted upon binding to Pab421
(lanes 4, 8, and 10 for P1, P3, P5 respectively). This
supershift is an indication that the P3 region in these mutants
are still capable of binding to the antibodies and that the
5   ability of the mutants to bind DNA in the absence of Pab421 was
not due to mutation at the P3 region. In fact, binding of the
antibodies Pab1801 outside of these C-terminal domain also
allowed a supershift of the complex. The mutant used in lanes
7 and 8 was mutated at the first 4 amino acid residue of the P3
10 region and that apparently does not affect the binding of Pab421
to the protein. The mutant used in lanes 5 and 6 was mutated at
the conserved amino acids of the last 4 residues in the P3 region
and that abolished the binding of Pab421 to the protein.
Mutation at the unconserved amino acids show no effect, the
15 proteins behaved essentially like the wild type. Thus, the
conformation of the mutant p53 proteins is switched to the active
form when the homology regions are destroyed by mutation.

        Examples 3-7 of the present invention demonstrate (1)
the ability of the mutant p53 to bind specific DNA sequences; (2)
20 the ability of the mutant p53 to bind to cyclin D1, D2 and D3;
(3) the ability of the mutant p53 to resist phosphorylation by
the cyclin A/cdk2 kinase at serine residue 315; (4) the ability
of the mutant p53 to activate transcription in vivo and (5) the
ability of the synthetic mutant p53 to induce apoptosis of tumor
25 cells when used in conjunction with the chemotherapeutic agent,
cisplatin.

                        EXAMPLE 4
        The present invention shows that p53 is able to bind
to cyclin D1, D2 and D3. The deregulated expression of cyclin
30 D is related to the development of various tumors including
leukemia and mammary tumors. To show the binding of p53 to
cyclin D, the corresponding recombinant baculoviruses were
created and co-infected into sf9 cells. The co-precipitation is
described in Figure 11 which shows that the mutant p53 protein
35 is able to bind specific DNA sequences. The p53 protein was
precipitated using a monoclonal antibody PAb 1801. As is shown
in the Figure 11, wildtype p53 (lane 1) was unable to bind to

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either of the three cyclin Ds when they were coexpressed *in vivo*
in infected sf9 cells (lanes 2, 3, 4). In contrast, the mutant
form of p53 in which the p3 and p5 regions were deleted (lane 5)
was able to bind to cyclin D1 (lane 2), D2 (lane 3), and D3
5   (lane 4).   Thus, the mutation of the Rb/p53 homology regions
resulted in the exposure of the functional domain of p53 such
that the p53 protein was able to interact with its binding target
DNA and proteins. The invention also shows that one such protein
was the protooncogene products cyclin D1, 2 and 3, the
10   deregulated expression is important for tumorigenesis.

EXAMPLE 5

In addition to the activation (exposure) of the
functional domain of p53, the conformational changes brought
about by the mutation of the Rb/p53 region also resulted in
15   specific change in the conformation of domain R in the C-
terminus.   Specifically, the serine residue 315 was not
phosphorylated by any of the cyclin-dependent kinases as the
wildtype. Figure 12 shows that the homology regions control the
exposure of the C-terminus of p53 so that specific sites are not
20   phosphorylated.

p53 proteins were expressed in bacteria. An aliquot
of the bacteria was labeled with ^{35}S-cysteine and methionine for
quantitation.   Equal amounts of purified p53 (Figure 12B) were
mixed with cyclin A: cdk 2 in a phosphorylation buffer containing
25   ^{32}P-ATP.   The phosphorylation reactions were carried out at room
temperature for 30 minutes.   The p53 proteins were then
precipitated with PAb1801. As can be seen in figure 12A, p53 was
heavily phosphorylated whereas the synthetic mutant P53 mutated
at the conserved aminoacid residues in domain P3 (lane 3C) or P5
30   (lane 5C) were not phosphorylated, if at all.   In contrast,
mutation at the unconserved amino acid residues even within the
P3 (3U) or the P5 (5U) regions had no effect on the
phosphorylation of the protein.   2-dimensional mapping of the
phospho-peptides confirmed that serine 315 is the predominant
35   r sidue that is phosphorylated.

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EXAMPLE 6

    To demonstrate the functional properties of the homology region mutants, the human lung carcinoma cell line H358 which lacks endogenous p53 proteins was cotransfected with the
5 p53 mutants and a reporter construct in which the CAT gene was under the control of a p53 CON. sequence containing a p53 consensus binding site. Figure 13 shows that the homology region mutants of p53 are transcriptionally active *in vivo*. As is shown in Figure 13A, p53 mutants of the P3 or P5 homology region at the
10 conserved aminoacids was active in promoting transcription from a promoter with a p53 binding site and the amount of transcriptional activation by the mutants was equal to or slightly higher than that of the wildtype. In contrast, the p4 mutant (which was mutated in the fifth conserved functional
15 domain) abolished the ability of p53 to bind cyclin D or DNA and was totally defective in promoting transcription. In the middle of Figure 13A, lane 0 represents the control in which only the vector without p53 inset was used. As expected, no transcription promotion can be detected). Equal amount of proteins were used
20 for all the p53 constructs as demonstrated in Figure 13B.

EXAMPLE 7

    The introduction of a wildtype p53 has been shown to be effective in the induction of apoptosis of tumor cells when chemotherapeutic agent treatment alone failed to stop tumor
25 growth. To show that the synthetic p53 mutant induced apoptosis, the lung carcinoma cell line H358 which is devoid of endogenous p53 protein due to an inactivating mutation was used. As a comparison, a batch of the same cell line was transfected with either the wildtype or the P4 mutant (which was shown to be
30 inactive by several criteria). Figure 14 shows that the synthetic p53 mutant induces apoptosis in the lung carcinoma cell line, H358. As is shown in Figure 14A, 14C, and 14E, approximately 5% of the H358 cells transfected with wildtype, P4 or P5 mutants respectively showed expression of the p53 proteins
35 in the nucleus by immunostaining with the anti-p53 monoclonal antibody PAb122. (Compare the dark staining of the cell nuclei and the lack of staining in the surrounding cells). After the

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cells were treated with the chemotherapeutic agent c-DDP
(cisplatin) for two weeks, most of the cells that expressed
wildtype (Figures 14B) or p5 mutant p53 (Figures 14F) underwent
apoptosis and disintegrated. In contrast, cells expressing the
5   p4 inactive mutant p53 (Figure 14D) continued to persist and
indeed proliferated to a greater number than before treatment.
Thus, the present invention demonstrated that the p5 mutant p53
induced apoptosis in conjunction with chemotherapeutic treatment.

EXAMPLE 8

10   Thus, the present invention demonstrates that the
p53/pRb homology regions (P1, P3 and P5) control the
conformations of the p53 protein in two different ways
simultaneously. First, the functional domain (domain F) which
is necessary for cell growth suppression and induction of
15   apotosis, is exposed when the Rb/p53 homology regions in the C-
terminus are modified or mutated. The exposure of the
functionally important central region of these mutant p53
proteins is shown by their enhanced ability to bind to specific
proteins and DNA sequences when compared to the wildtype protein.
20   Secondly, the mutation of the homology regions also brought about
conformational changes at the C-terminal regions. That is,
domain R became hidden such that the ability of p53 to be
phosphorylated at serine #315 is abolished. In addition,
exposure of the C-terminal domains needed for double stranded
25   DNA/RNA annealing activity is controlled by the P3 region, since
blocking the P3 region with PAb421 allows translation of the p53
mRNA. This annealing activity prevents newly synthesized p53
mRNA from being translated. Therefore, mutations at any of the
P1, P3 or P5 regions in the conserved amino acids resulted in a
30   p53 conformation in which various activities known to be
important to the growth suppressor function of the proteins are
unmasked. The conformational change was not specific for any one
particular effect.

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EXAMPLE 9

Alteration in the control of the phosphorylation of Rb proteins
mutated at the homology region

        There are several ways to distinguish between the
5   active and inactive conformations of the pRb protein. Active Rb
    protein is underphosphorylated and can suppress the growth of the
    cell.   Inactive Rb protein is heavily phosphorylated.   If the
    homology domain affects the active and inactive conformation of
    pRb, mutation at the P1, P3, P5 regions should affect the ability
10 of the protein to suppress cell growth and its phosphorylation
    accordingly. The P1, P3, and P5 regions of pRb were separately
    mutated at the conserved amino acid residues and the ability of
    the mutants to suppress cell growth examined. All mutants were
    able to suppress the growth of the transfected cells.   The
15 ability of the Rb mutant m89-0, which is mutated at the conserved
    amino acid residues at the P5 region, to suppress the growth of
    normal and cancer cells is given in two Examples below. Since
    the activity of pRb is regulated by phosphorylation, if the
    conserved region is for the maintenance of pRb in an inactive
20 conformation, mutation of the P5 region should have an inhibitory
    effect on the phosphorylation of the mutant pRb such that the
    protein is active.

        There are cell lines which are devoid of pRb expression
    due to mutation of both alleles of RB-1 and these cell lines have
25 also incurred other mutations which render them resistant to the
    suppression effect of a transfected Rb gene.   The cervical
    carcinoma cell line C33A and a subpopulation of the Saos-2 are
    two such cell lines. These cell lines therefore would allow the
    mutant proteins to be expressed and the cells remain viable,
30 allowing the extraction of abundant amounts of the protein for
    examination of the phosphorylation status. The P5 mutant HuAcPr-
    1-neo-P5 C, mutated at the conserved amino acid residues at the
    P5 region, was therefore transfected into these cells and stable
    cell lines were obtained by single cell purification. The
35 phosphorylation patterns of the mutant pRb isolat d from the
    purified mutant cell line Saos-2#89 was compared to that of the
    wild type pRb extracted from the wildtype Rb transfected and

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purified cell line Saos-2#84. Immunoprecipitation of pRb from
S phase Saos-2#84 (wildtype) and Saos-2#89 (mutant) showed that
the wildtype pRb was heavily phosphorylated whereas the mutant
pRb was underphosphorylated. This suggests that the P5 mutant
5 pRb remains in the active conformation even when the cells were
in the S phase. Thus, the P5 mutant assumes a conformation which
is resistant to phosphorylation at particular sites. It should
be noted that the protein was still phosphorylated at other
functionally unimportant sites.

10   Since one-dimensional analysis by SDS-PAGE only
revealed that the protein was underphosphorylated but not the
nature   of   the   underphosphorylation,   two-dimensional
phosphopeptide analyses of the ^{32}P-labelled pRb using two
different proteases, trypsin and chymotrypsin, was performed.
15 A comparison of the 2-Dimensional maps revealed that there are
2 sites, S10a and S10b, that are consistently unphosphorylated
in the mutant as compare to the wild type (Figure 4A). To show
that the difference in the phosphorylation pattern was due to an
intrinsic difference in the conformation of the proteins instead
20 of variation in *in vivo* enzyme specificity, the *in vitro*
phosphorylation patterns of the proteins were compared. The
wildtype and mutant pRb were phosphorylated using a kinase
preparation that consist of cyclin associated kinases. The *in
vitro* labelling was dependent on the cyclin associated kinase as
25 antibodies against the kinase blocked the phosphorylation. The
in vitro phosphorylated pRb were analyzed by 2-Dimensional
peptide mapping and as shown in Figure 4B, the same
phosphorylation sites were not used in the mutant. Therefore,
even with the supply of abundant amounts of enzymes and optimum
30 phosphorylation conditions in which the wildtype pRB was
phosphorylated to completion, the mutant pRb was completely
resistant to the phosphorylation sites S10a and S10b. The
variation in the phosphorylation of S10a and S10b is therefore
due to an intrinsic difference in the conformation of the
35 wildtype and the mutant proteins instead of variation in *in vivo*
enzyme specificity.

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EXAMPLE 10

Rb proteins mutated at the homology region are active
Correlation of phosphorylation at S10a and S10b with pRb activity

The data provided below demonstrates that the variation
5   in the phosphorylation at S10a and S10b correlates with the
ability of pRb to bind SV40 large T antigen. For *in vitro*
phosphorylation studies, Rb protein was isolated from sf9 cells
infected with a recombinant Rb-Baculovirus in which the
polyhedron gene was partially replaced with the Rb gene. It was
10 noticed that, under high infection titer conditions, although
the majority of the Rb protein extracted was rendered incapable
of binding to the SV40 large T by phosphorylation, a small
proportion of Rb protein that remained bound to large T. This
binding is shown in Figure 5. The Rb protein and the SV 40 large
15 T were obtained from ^{35}S-labeled SF9 cells infected with the
corresponding bacculoviruses and kinased in vitro. Lane 1 shows
that the Rb proteins extracted consisted of underphosphorylated
as well as hyperphosphorylated forms and some of the proteins
bound to large T.   Large T could bind only the
20 underphosphorylated form of the RB protein (lane 2 and lane 3).
Phosphorylation of this large T:Rb complex with different amounts
of Rb kinase (lanes 3 through 6) released the majority of the Rb
protein from the large T. This form of the RB proteins can be
termed RB-E (E = exposed critical site). However, approximately
25 20-30% of the Rb protein (termed RB-H) (H = hidden critical site)
remained tightly bound to large T and the whole complex could be
brought down by anti-large T antibody.   Most importantly,
although increasing amount of the Rb kinase could phosphorylate
the Rb protein to its slowest migrating form (compare lane 4
30 through 6 and lanes 8 through 10), the hyperphosphorylated Rb
protein remained bound to the large T. That the precipitation
of these Rb proteins was not due to nonspecific absorption to
the protein A agarose is shown by comparison of lane 6 with lane
2.   Approximately 70% of the Rb protein was released from the
35 large T. This released Rb protein was not due to a nonspecific
degradation of the protein as shown by the fact that if the
reaction was precipitated with anti-Rb antibodies, all of the Rb

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proteins w re accounted for (as shown in lanes 8, 9 and 10). The ability of the various forms of Rb protein to bind to large T is also shown in lanes 8, 9 and 10, in which a smaller amount of large T was brought down by the Rb protein.

5   These results are consistent with the presence of two classes of phosphorylation sites on the Rb protein, with one being critical for the inactivation of the Rb protein so that it cannot bind to large T, whereas the others can be phosphorylated without affecting the binding. There are 17 potential

10 phosphorylation sites (S/TPXX) for the Rb kinase complex. To demonstrate that the phosphorylation sites are important for the inactivation of the Rb protein, a 2-D mapping analysis was performed on the Rb protein that remains bound to the SV40 large T (RB-H) and on those that are free from the SV40 large T after

15 phosphorylation (RB-E). There must be a difference in the phosphorylation sites between these two forms of Rb protein if the disassociation of Rb from large T is a result of phosphorylation. As is shown in Figure 6, S10a and S10b were found not to be phosphorylated in the pRb that remain bound to

20 the large T protein.

           Mutation at the conserved amino acids at the P5 region renders the protein an active conformation. The phosphorylation at S10a and S10b is a function of the cell cycle and correlates with the ability of the cell to proliferate.

25   EXAMPLE 11
Phosphorylation at S10a and S10b of pRb and cell growth
           The data provided below demonstrates that the variation in the phosphorylation at S10a and S10b is regulated in a cell cycle dependent manner. It is well documented that exposure of

30 cells, normal or cancerous, to TGF-β frequently lead to growth arrest. A role of pRb in the suppression of cell growth has previously been described. To show the role of TGF-β on the phosphorylation of pRb, the breast carcinoma cell line MDAMB231 which can be growth arrested by treatment with TGF-β was

35 employed. The proliferation status of the cells were monitored by tritiated thymidine uptake and the phosphorylation pattern of the ^{32}P-labelled pRb analyzed by 2-Dimensional peptide mapping.

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Significantly, the pRb from cells growth arrested by TGF-β were
not phosphorylated at S10a and S10b (Figure 7). However, when
the cells were released from the TGF-β, S10a and S10b were again
phosphorylated. Thus, the pRb protein which is active in growth
5   arrested cells were not phosphorylated at S10a and S10b. That
this reversible phosphorylation was not just specific to the
growth suppression effect of TGF-β was shown by the fact that
similar phosphorylation pattern of S10a and S10b was also found
in pRb isolated from cells growth arrested by deprivation of
10 serum. Upon refeeding with either serum or growth factors, S10a
and S10b was once again phosphorylated. In addition, S10a and
S10b was not phosphorylated in pRb isolated from cells growth
arrested by retinoic acid. Therefore, the functionally critical
S10a and S10b sites in the pRb protein can undergo reversible
15 phosphorylation under different growth conditions.

                        EXAMPLE 12

Mutants of the Rb gene and the p53 gene

        Thus, for both pRb and p53, mutation of the homology
region resulted in the proteins assuming conformations that
20 render them active. Examples of the conformationally active
mutants of Rb and p53, and their uses in suppression of cell
growth, are given below. For the Rb protein, two classes of
mutants are described, (1) mutants in the regulatory or homology
region, and (2) mutants in the phosphorylation sites. For the
25 p53 protein, mutants of the homology regions are described.

        Phosphoamino acid analysis of hyperphosphorylated Rb
proteins revealed that the amino acids serine and threonine are
phosphorylated. There are 79 serine and 55 threonine residues
in the Rb protein. Of these, 17 are found in the motif (S/TPXX),
30 a potential phosphorylation site for the cdc2 kinase. The cdc2
kinase, and related kinases, are known to be able to
phosphorylate the Rb protein.

        There appear to b two classes of phosphorylation sites
on the Rb protein, with one being critical for the inactivation
35 of the Rb protein so that it cannot bind to large T, whereas the
others can be phosphorylated without affecting the binding.
Because there are at least 17 potential phosphorylation sites

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(S/TPXX) for the Rb kinase complex, several may be important for the inactivation of the Rb protein. These 17 phosphorylation sites are T005, S230, S249, T252, T356, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821, T826, where T and S
5 denotes threonine and serine, respectively, and the number that follows denote the residue number of the amino acid in the Rb protein.

The mutated retinoblastoma gene is preferably mutated by one of two different ways, both of which affect the ability
10 of the protein to be phosphorylated. First, the gene may be mutated by changing amino acids in conserved homology regions of the Rb gene. Preferably, the conserved homology region of the Rb gene is selected from the group consisting of P1, P3, and P5, as described herein. Representative examples of mutated Rb genes
15 that encodes for a mutated Rb protein is shown in Seq. ID. No. 1 and 2. A person having ordinary skill in this art, with the knowledge of the importance of the conserved homology domains to the phosphorylation of the protein, could easily construct other mutated Rb genes that encode for other desirable mutated Rb
20 proteins.

A second way of preventing the phosphorylation and thus inactivation of the Rb protein is when said gene is mutated by changing amino acids in the phosphorylation sites. Preferably, the phosphorylation sites are selected from the group consisting
25 of T005, S230, S249, T252, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821 and T826. In the abbreviations for the phosphorylation sites above, S refers to serine, T refers to threonine, and the number refers to the placement of the amino acid in the sequence of the Rb protein. For the phosphorylation
30 mutants disclosed herein, each of the threonine or serine residues was mutated into alanine in individual plasmids. As stated before, there are at least 2 classes of phosphorylation sites, one of which is the functionally important for the inactivation of pRb. Therefore one or more of the mutants
35 created above should n gate the phosphorylation of the mutant pRb and prevent its inactivation.

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In other embodiments of the present invention, there are also provided plasmids comprising the DNA encoding for mutated Rb proteins. In addition, also provided is a plasmid adapted for expression in a recombinant cell comprising the DNA

5   encoding for a mutated Rb protein of Seq. ID. No. 1 and regulatory elements necessary for expression of the cDNA in the cell and a plasmid adapted for expression in a recombinant cell comprising the DNA encoding for a mutated Rb protein of Seq. ID. No. 2 and regulatory elements necessary for expression of the

10   cDNA in the cell.

EXAMPLE 12

Mutants of the p53 gene

In another embodiment of the present invention, the mutated growth suppressor gene is a mutated p53 gene.

15   Preferably, the p53 gene is mutated by changing amino acids in conserved homology regions of the p53 gene (underlined in Table I above). Similarly, the conserved homology region of the p53 gene is selected from the group consisting of P1, P3 and P5. Mutants can be consisting of mutations at either P1, P3, or P5,

20   singularly or in any combinations (including mutations of all three regions). In addition, mutants in which the C-terminal amino acid residues 295-393 were deleted were still active in binding to the specific DNA sequence.

EXAMPLE 13

25   Use of pRb and p53 mutants for cell growth suppression

The present invention also discloses novel transfected cells, e.g., the transfected cell comprising the isolated DNA molecule of Claim 1 which encodes a mutated Rb protein. Also provided by the present invention are stably transfected cells

30   expressing a mutated Rb gene encoding for a mutated Rb protein. Preferably, the transfected cells of present invention contain the mutated Rb gene comprising a nucleic acid sequence encoding for a mutated Rb protein shown in Seq. ID No. 1 or Seq. ID No. 2. In addition, the present invention also provides a stably

35   transfected cell expressing a mutated p53 gene encoding for a mutated p53 protein.

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The present invention also provides a method for the treatment of pathological cell proliferative diseases comprising administration to a non-cancerous proliferating cell of the DNA molecule of a mutated growth suppressor gene. The molecule administered, in one embodiment, is a mutated Rb or p53 gene. The gene is mutated by changing amino acids in conserved homology regions of the Rb gene or said gene is mutated by changing amino acids in phosphorylation sites. The conserved homology regions and the phosphorylation sites are the same as described above.

Generally, this method may be used to treat any cell proliferative disease. Representative examples of non-cancerous cell proliferative disease include psoriasis, benign proliferative skin diseases, ichthyosis, papilloma, basal cell carcinoma, squamous cell carcinoma, fibroproliferative diseases, vasoproliferative diseases and dermatoproliferative diseases.

The present invention also provides a method of treating malignant cell diseases in individuals comprising administration into a proliferating cancer cell of a DNA molecule of a mutated Rb or p53 gene. These mutated genes are constructed as described above.

Generally, the method of treating malignant cell diseases is similar to the treatment of non-cancerous cell proliferative diseases. Representative examples of diseases caused by proliferating cancer cells, include but not limited to, osteosarcoma, fibrosarcoma, retinoblastoma, carcinoma of the breast, bladder, cervix, lung, colon, ovary, kidney, pancreas, and prostate.

According to the method of the present invention, one can manipulate the cellular proliferative process by the introduction of a mutant RB or p53 protein to prevent or inhibit abnormal proliferation in a wide variety of cell proliferative diseases. The manipulation of the proliferative process may be accomplished by introducing into a target proliferating cell a DNA construct which encodes a mutant RB or p53 protein element.

The present invention also provides a method for th treatment of focal cell proliferative diseas s comprising administration of the mutated Rb or p53 gene to proliferating

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cells.   Generally, the proliferating cell may be the cause of
such   representative   diseases   as   fibroproliferative,
vasoproliferative and neoplastic diseases in the eye.

5   In another embodiment, the phosphorylation site of the
Rb gene is mutagenized before introduction into a cell.   There
are 17 potential phosphorylation sites on the Rb protein.
Mutation of the serine or threonine coding sequences in the Rb
cDNA into alanine or valine or others would therefore lead to the
production of a permanently active Rb protein which cannot be
10   inactivated by phosphorylation.   That is, the host cell will not
be able to inactivate the Rb protein by phosphorylation.
Introduction of such a mutated Rb gene into a cell will therefore
lead to growth arrest.   All these mutants have been subcloned in
the human β-actin promoter vector.   For subcloning of the Rb
15   cDNA, the entire open reading frame was ligated to a synthetic
linker containing the BamH1 site at the 5' end and a synthetic
linker containing AATAAA and a BamH1 site at the 3' end.   The
resulting BamH 1 fragment was subcloned into the BamH 1 site of
the vector and the clone with the correct orientation selected.

20   EXAMPLE 14

Preparation of the p5 mutants m89-0 and m88-0

             To   create   the   m89-0   mutant,   a   pair   of   DNA
oligonucleotide primers (Pm1 and QM1) containing the region 'P5'
to be mutated and a pair of oligonucleotides flanking the region
25   'P5' (P3 and Q3) were synthesized using the AB1 PCR-mate
oligonucleotide   synthesizer   according   to   manufacturer
instruction.   Using the wild type Rb cDNA as the template, 40
cycles of polymerase chain reactions (PCR) were performed with
the primers P3 and Qm1.   In a second tube, 40 cycles of
30   polymerase chain reactions were performed with the primers Pm1
and Q3.   After the completion of the reaction, a small aliquot
of the reaction product was taken from the first tube and 80
cycles of PCR were performed in the presence of excess P3 primer.
Similarly, 80 cycles of PCR were performed in the presence of
35   excess Q3 primer in the second tube.   The predominately single
strand reaction products from these two tubes were then combined
and allowed to primer xtend for 20 cycles.   The resulting double

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stranded product, now containing the mutated P5 region were
subcloned into the Rb expression plasmid HuBAcpr-1-neo-PQ (m89-0)
at the Mlu 1 and Hind 3 sites. The resulting plasmid has a
mutated P5 region shown below with the mutated amino acids
5 underlined. K870 and H890 refer to the lysine and histidine
amino acid residues at position 870 and 890 respectively in the
Rb protein. m88-0 was created similarly.

Wildtype Rb:- K_{870}PLKKLRFDIEGSDEADGSKH$_{890}$
Mutant m89-0 Rb:- K_{870}PLKKLRFDIEASAEVDASIH$_{890}$
10 Mutant m88-0 Rb:- K_{870}LLIKPRYDTEGSDEADGSKH$_{890}$

EXAMPLE 15

Effect of Transfected Plasmids on the Human Fibroblast Cell Line

The effect of retinoblastoma cDNA on cell growth was
seen as the mutant m89-0 was transfected into the normal human
15 fibroblast cell line WS1. The gene for the SV40 large T antigen
was used as a control for the transfection. A plasmid, pPVUO-
Neo, which contains the gene for the SV40 large T antigen and a
neomycin resistance gene was then transfected in order to monitor
the efficiency of transfection. In addition, an inactive mutant
20 form of the Rb gene, "HuBAcpr-1-neo-P16" was used as a control.
For transfection, 100 μg of each of the plasmid DNA were mixed
with 107 exponentially grown WS1 cells in a final volume of 0.8
ml of RPM1 1640 medium (Gibco) plus 10% FCS in an electroporation
chamber unit Cell-PoratorTM (BRL). Electroporation was done at
25 200 volt and 1180 μF. The electroporated cells were allowed to
recover at room temperature for 10 minutes and were then diluted
in RPM1 1640 plus 10% fetal calf serum (FCS) and plated out in
60 mm dishes at a cell density of 2 X 104 cells/cm2. The cells
were allowed to attach and grow in the same medium for two days.
30 Thereafter, the medium was changed to RPM1 1640 + 10% FCS +
15ug/ml G418 (GIBCO). Every three days, duplicate dishes were
tak n for histoimmunoch mical staining using either a rabbit
polyclonal anti-Rb protein antibodies RB1-ABA1 (Figure 8A), or
with the mouse monoclonal anti-SV40 large T antigen antibody Pab
35 101 (Figure 8B). As can be seen from Figure 8A, 13 days after

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transfection and selection in G418 medium, the WS1 cells expressing the transfected Rb cDNA plasmid m89-0 stained intensely with the anti-Rb protein antibodies. However, the cells that expressed the Rb protein remained as single cells and
5 did not divide. In contrast, cells that expressed the transfected SV40 large T antigen (SVLT) continued to divide into colonies as shown by the group of cells stained positive with the mouse anti SVLT antibody in Figure 8B. Thus, over expression of the m89-0 Rb cDNA in a cell led to complete arrest of cell growth
10 of the normal cells WS1. Cells transfected with the inactive form of Rb HuβAcpr-1-neo-P16 were not suppressed but divided into colonies.

EXAMPLE 16

Effect of Transfected Plasmids on a Bladder Carcinoma Cell Line
15 The mutant m89-0 and HuβAcpr-1-neo-P16 plasmids were also transfected into the human bladder carcinoma cells TCCSUP which has no endogenous Rb protein using the same procedures described above. The plasmids used, the methodology for transfection and immunostaining are identical to those described
20 above.

As can be seen in Figure 9A, cells expressing the transfected m89-0 cDNA failed to divide. In contrast, cells expressing the transfected HuBAcpr-1-neo-P16 divided to form colonies (Figure 9B). Overexpression of the m89-0 Rb cDNA led
25 to severe retardation of growth of the tumor cells in vitro.

EXAMPLE 17

In addition to the mutants at the regions homologous between Rb and p53, mutations in the p53 protein. Table II lists the partial amino acid sequence of the wild type p53 and
30 conserved amino acid mutants prepared. In addition, Table II lists the DNA sequence corresponding to the mutant amino acid sequence. To prepare the mutants, two primers were synthesized and ann aled together. PCR was carried out as described above. As shown in above, the alteration P1, P3 and P5 regions lead to
35 a change in the conformation of the p53 protein such that it is now capable of binding to its target DNA sequence directly.

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TABLE II

<u>p53 MUTANTS</u>

P1   WIldTYPE: ELNEALELK

    CONSERVED a.a. MUTANT (P53-Con P1):   KLYEDFEIE

5   (1) 5' GC TCC GAG ATG TTC CGA AAG CTG TAT GAG GAC TTG GAA ATC GAG

GAT GCC CAG GCT GGG 3'

    (2) 3' CG AGG CTC TAC AAG GCT TTC GAC ATA CTC CTG AAC CTT TAG CTC

CTA CGG GTC CGA CCC 5'

    P3   WILDTYPE: KKGQSTSRH

10   CONSERVED aa MUTANT (P53-Con P3):   NEWFSTARD

    (1) 5' AGC CAC CTG AAG TCC AAC GAG TGG TTC TCT ACC GCC CGC GAT

AAA AAA CTC ATG TTC AAG AC 3'

    (2) 3' TCG GTG GAC TTC AGG TTG CTC AGG AAG AGA TGG CGG GCG CTA

TTT TTT GAG TAC AAG TTC TG 5'

15   P5   WILDTYPE: KKLMFKTEGPDSD

    Conserved a.a. MUTANT (P53-Con P5):   NEVMWKTKWPDAH

    (1) 5'GTCTACCTCCCGCCAT AACGAA GTCATG TGGAAG ACAAAA TGGCCT GACGCA

CAC TGACATTCTCCACTTCTT 3'

    (2) 3'CAGATGGAGGGCGGTA TTGCTT CAGTAC ACCTTC TGTTTT ACCGGA CTGCGT

20   GTG ACTGTAAGAGGTGAAGAA 5'

EXAMPLE 18

<u>Generation of the recombinant retrovirus containing a mutant Rb gene</u>

        A Hind III/Bam HI fragment containing the entire coding
25   region of the mutant Rb#89 is subcloned into the Hind III/BgIII
sites in a retroviral vector. The resulting plasmid vectors are
transfected into the cell line Psi-2. The medium used to culture
the transfected cells is infected into another cell line PA317.
Cells that produce the recombinant virus were selected in G418
30   and the r trovirus collected from the medium is concentrated and
purified through a sucrose gradient. Mutants of the p53 gene is
tested in an identical manner.

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EXAMPLE 19
Effect of the G1NaSvRb89 virus on cell proliferation in the eyes

Experimental Proliferative Vitreous Retinopathy (PVR) in rabbits is induced by injection of rabbit fibroblasts and induction of local wound. This procedure consists of inducing a wound at limbus and injecting 2×10^5 rabbit fibroblasts into the vitreous under general anesthesia. Post-operatively, the rabbit will receive antibiotic containing local ointment.

Recombinant retroviruses with the mutant Rb or p53 gene are injected into the vitreous (2×10^5 cells/rabbit) with the animal under general anesthesia. Initially, local tolerance of viruses is tested by assessing the maximum dosage of virus (concentration of the virus to be used and the volume of injecting virus). Retroviruses have already been used for injection beneath the retina in mice without reported side effects (Turner and Cepko, *Nature* 328:132, 1987). The maximum dosage that would not cause side effects will be employed for all subsequent treatment.

The growth of cells *in vivo* is monitored by photography. The grade of retina detachment is compared with the controls where only a retrovirus containing a 1-Galactosidase gene was injected. Additionally, the growth of cells *in vivo* is monitored by labelling intra-vitum with BromodeoxyUridine. One hour before euthanasia, the rabbit is given 50 mg/kg BrdU I.V. to label the proliferating cells. After euthanasia, the cells are analyzed for BrdU incorporation into their DNA by immunostaining with antibodies to BrdU. Simultaneously, immunostaining of the cells with antibodies against the Rb or p53 protein or the 1-Galactosidase protein are also performed to assess the efficiency of the infection.

The optimum time for the retrovirus to suppress the growth of the injected cells is assessed by injecting the virus at various time after induction of PVR. The effect of the vitreous on the infection efficiency in rabbit fibroblasts is also determined. The time delay for adding these viruses on rabbit fibroblasts is also determined. The time delay for adding these viruses on rabbit fibroblasts that stimulated with

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fibroblast growth factors to increase the growth rate is also determined.

All patents and publications mentioned in this specification are indicative of the levels of those skilled in
5   the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the
10 present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred
15 embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

     (i) APPLICANT: Fung, Yuen Kai

     (ii) TITLE OF INVENTION: Mutants of the Rb and p53 Genes
                                           and Uses Thereof

     (iii) NUMBER OF SEQUENCES: 15

     (iv) CORRESPONDENCE ADDRESS:

          (A) ADDRESSEE: James F. Weiler, Attorney-at-Law

          (B) STREET: One Riverway, Suite 1560

          (C) CITY: Houston

          (D) STATE: Texas

          (E) COUNTRY: USA

          (F) ZIP: 77056

     (v) COMPUTER READABLE FORM:

          (A) MEDIUM TYPE: DS, HD 1.44 Mb/1.44 Mo

          (B) COMPUTER: IBM PC compatible

          (C) OPERATING SYSTEM: PC-DOS/MS-DOS

          (D) SOFTWARE: WordPerfect 6.0

     (vi) CURRENT APPLICATION DATA:

          (A) APPLICATION NUMBER:

          (B) FILING DATE:

          (C) CLASSIFICATION:

     (viii) ATTORNEY/AGENT INFORMATION:

          (A) NAME: Weiler, James F.

          (B) REGISTRATION NUMBER: 16,040

          (C) REFERENCE/DOCKET NUMBER: D-5382 CIP

     (ix) TELECOMMUNICATION INFORMATION:

          (A) TELEPHONE: 713-626-8646

          (B) TELEFAX: 713-963-5853

(2) INFORMATION FOR SEQ ID NO:1:

     (i) SEQUENCE CHARACTERISTICS:

          (A) LENGTH: 20

          (B) TYPE: amino acid

          (C) STRANDEDNESS:

          (D) TOPOLOGY: linear

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```
     (ii) MOLECULE TYPE:
          (A) Description: peptide
    (iii) HYPOTHETICAL: No
     (iv) ANTISENSE: No
 5   (v)  FRAGMENT TYPE: internal fragment
     (vi) ORIGINAL SOURCE:
          (B) STRAIN:
          (C) INDIVIDUAL ISOLATE:
          (D) DEVELOPMENTAL STAGE:
10        (F) TISSUE TYPE:
          (G) CELL TYPE:
          (H) CELL LINE:
     (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
          Leu Leu Ile Lys Pro Arg Tyr Asp Thr Glu Gly Ser Asp Glu
15        1               5                   10
          Ala Asp Gly Ser Lys His
          15                  20


 (3) INFORMATION FOR SEQ ID NO:2:
      (i) SEQUENCE CHARACTERISTICS:
20        (A) LENGTH: 20
          (B) TYPE: amino acid
          (C) STRANDEDNESS:
          (D) TOPOLOGY: linear
     (ii) MOLECULE TYPE:
25        (A) Description: peptide
    (iii) HYPOTHETICAL: No
     (iv) ANTISENSE: No
     (v)  FRAGMENT TYPE: internal fragment
     (vi) ORIGINAL SOURCE:
30        (B) STRAIN:
          (C) INDIVIDUAL ISOLATE:
          (D) DEVELOPMENTAL STAGE:
          (F) TISSUE TYPE:
          (G) CELL TYPE:
35        (H) CELL LINE:
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(xi)  SEQUENCE DESCRIPTION: SEQ ID NO:2:
      Pro Leu Lys Lys Leu Arg Phe Asp Ile Glu Ala Ser Ala Glu
      1                   5                       10
      Val Asp Ala Ser Ile His
      15                      20


(4)  INFORMATION FOR SEQ ID NO:3:
     (i)   SEQUENCE CHARACTERISTICS:
           (A)  LENGTH: 20
           (B)  TYPE: amino acid
           (C)  STRANDEDNESS:
           (D)  TOPOLOGY: linear
     (ii)  MOLECULE TYPE:
           (A)  Description: peptide
     (iii) HYPOTHETICAL: No
     (iv)  ANTISENSE: No
     (v)   FRAGMENT TYPE: internal fragment
     (vi)  ORIGINAL SOURCE:
           (B)  STRAIN:
           (C)  INDIVIDUAL ISOLATE:
           (D)  DEVELOPMENTAL STAGE:
           (F)  TISSUE TYPE:
           (G)  CELL TYPE:
           (H)  CELL LINE:
     (ix)  FEATURE:
           (A)  OTHER INFORMATION:    Represents amino acids 871-
                                      890  of  the  retinoblastoma
                                      protein.
     (xi)  SEQUENCE DESCRIPTION: SEQ ID NO:3:
           Pro Leu Lys Lys Leu Arg Phe Asp Ile Glu Gly Ser Asp Glu
           1                   5                       10
           Ala Asp Gly Ser Lys His
           15                      20


(5)  INFORMATION FOR SEQ ID NO:4:
```

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    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 9

        (B) TYPE: amino acid

        (C) STRANDEDNESS:

5   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: peptide

    (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

10   (v) FRAGMENT TYPE: internal fragment

    (vi) ORIGINAL SOURCE:

        (B) STRAIN:

        (C) INDIVIDUAL ISOLATE:

        (D) DEVELOPMENTAL STAGE:

15   (F) TISSUE TYPE:

        (G) CELL TYPE:

        (H) CELL LINE:

    (ix) FEATURE:

        (A) OTHER INFORMATION: Represents amino acids 184-

20   192 of the retinoblastoma

                                protein.

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

        Glu Ile Asn Ser Ala Leu Val Leu Lys

        1   5

25 (6) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 9

        (B) TYPE: amino acid

        (C) STRANDEDNESS:

30   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: peptide

    (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

35   (v) FRAGMENT TYPE: internal fragment

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(vi) ORIGINAL SOURCE:
    (B) STRAIN:
    (C) INDIVIDUAL ISOLATE:
    (D) DEVELOPMENTAL STAGE:
5   (F) TISSUE TYPE:
    (G) CELL TYPE:
    (H) CELL LINE:
(ix) FEATURE:
    (A) OTHER INFORMATION: Represents amino acids 343-
10   351 of the p53 protein.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
    Glu Leu Asn Glu Ala Leu Glu Leu Lys
    1   5

(7) INFORMATION FOR SEQ ID NO:6:
15 (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 9
    (B) TYPE: amino acid
    (C) STRANDEDNESS:
    (D) TOPOLOGY: linear
20 (ii) MOLECULE TYPE:
    (A) Description: peptide
(iii) HYPOTHETICAL: No
(iv) ANTISENSE: No
(v) FRAGMENT TYPE: internal fragment
25 (vi) ORIGINAL SOURCE:
    (B) STRAIN:
    (C) INDIVIDUAL ISOLATE:
    (D) DEVELOPMENTAL STAGE:
    (F) TISSUE TYPE:
30 (G) CELL TYPE:
    (H) CELL LINE:
(ix) FEATURE:
    (A) OTHER INFORMATION: Represents amino acids 245-
                             262 of the retinoblastoma
35   protein.

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        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
             Arg Arg Gly Gln Asn Arg Ser Ala Arg
             1   5

    (8) INFORMATION FOR SEQ ID NO:7:
5   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 9
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear
10   (ii) MOLECULE TYPE:
            (A) Description: peptide
      (iii) HYPOTHETICAL: No
       (iv) ANTISENSE: No
        (v) FRAGMENT TYPE: internal fragment
15   (vi) ORIGINAL SOURCE:
            (B) STRAIN:
            (C) INDIVIDUAL ISOLATE:
            (D) DEVELOPMENTAL STAGE:
            (F) TISSUE TYPE:
20   (G) CELL TYPE:
            (H) CELL LINE:
       (ix) FEATURE:
            (A) OTHER INFORMATION: Represents amino acids 372-
                                      380 of the p53 protein.
25   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
             Lys Lys Gly Gln Ser Thr Ser Arg His
             1   5

    (9) INFORMATION FOR SEQ ID NO:8:
        (i) SEQUENCE CHARACTERISTICS:
30   (A) LENGTH: 14
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE:
35   (A) Description: peptide

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```
      (iii) HYPOTHETICAL: No
      (iv)  ANTISENSE: No
      (v)   FRAGMENT TYPE: internal fragment
      (vi)  ORIGINAL SOURCE:
 5          (B) STRAIN:
            (C) INDIVIDUAL ISOLATE:
            (D) DEVELOPMENTAL STAGE:
            (F) TISSUE TYPE:
            (G) CELL TYPE:
10          (H) CELL LINE:
      (ix) FEATURE:
            (A) OTHER INFORMATION:   Represents amino acids 873-
                                     886 of the retinoblastoma
                                     protein.
15    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
            Lys Lys Leu Arg Phe Asp Ile Glu Gly Ser Asp Glu Ala Asp
            1                   5                   10


   (10) INFORMATION FOR SEQ ID NO:9:
      (i) SEQUENCE CHARACTERISTICS:
20          (A) LENGTH: 13
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
            (D) TOPOLOGY: linear
      (ii) MOLECULE TYPE:
25          (A) Description: peptide
      (iii) HYPOTHETICAL: No
      (iv)  ANTISENSE: No
      (v)   FRAGMENT TYPE: internal fragment
      (vi)  ORIGINAL SOURCE:
30          (B) STRAIN:
            (C) INDIVIDUAL ISOLATE:
            (D) DEVELOPMENTAL STAGE:
            (F) TISSUE TYPE:
            (G) CELL TYPE:
35          (H) CELL LINE:
```

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(ix) FEATURE:

    (A) OTHER INFORMATION: Represents amino acids 381-393 of the p53 protein.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

    Lys Lys Leu Met Phe Lys Thr Glu Gly Pro Asp Ser Asp

    1   5   10

(11) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 9

        (B) TYPE: amino acid

        (C) STRANDEDNESS:

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: peptide

    (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

    (v) FRAGMENT TYPE: internal fragment

    (vi) ORIGINAL SOURCE:

        (B) STRAIN:

        (C) INDIVIDUAL ISOLATE:

        (D) DEVELOPMENTAL STAGE:

        (F) TISSUE TYPE:

        (G) CELL TYPE:

        (H) CELL LINE:

    (ix) FEATURE:

        (A) OTHER INFORMATION: Represents mutant protein of amino acids 343-351 of the p53 protein.

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

        Lys Leu Tyr Glu Asp Phe Glu Ile Glu

        1   5

(12) INFORMATION FOR SEQ ID NO.11:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 59

        (B) TYPE: nucleic acid

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        (C) STRANDEDNESS: double stranded

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: other

5   (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

    (vi) ORIGINAL SOURCE:

        (B) STRAIN:

        (C) INDIVIDUAL ISOLATE:

10   (D) DEVELOPMENTAL STAGE:

        (F) TISSUE TYPE:

        (G) CELL TYPE:

        (H) CELL LINE:

    (ix) FEATURE:

15   (A) OTHER INFORMATION: Represents   nucleic   acids

                                encoding mutant protein of

                                sequence ID No. 10.

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO.11:

GCTCCGAGAT GTTCCGAAAG CTGTATGAGG ACTTGGAAAT CGAGGATCGG   50

20   CAGGCTGGG   59

  (13) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 9

        (B) TYPE: amino acid

25   (C) STRANDEDNESS:

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

        (A) Description: peptide

    (iii) HYPOTHETICAL: No

30   (iv) ANTISENSE: No

    (v) FRAGMENT TYPE: internal fragment

    (vi) ORIGINAL SOURCE:

        (B) STRAIN:

        (C) INDIVIDUAL ISOLATE:

35   (D) DEVELOPMENTAL STAGE:

        (F) TISSUE TYPE:

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       (G) CELL TYPE:

       (H) CELL LINE:

    (ix) FEATURE:

       (A) OTHER INFORMATION: Represents mutant protein of

5   amino acids 372-380 of the

                                         p53 protein.

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

       Asn Glu Trp Phe Ser Thr Ala Arg Asp

       1   5

10   (14) INFORMATION FOR SEQ ID NO.13:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 62

       (B) TYPE: nucleic acid

       (C) STRANDEDNESS: double stranded

15   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE:

       (A) Description: other

   (iii) HYPOTHETICAL: No

    (iv) ANTISENSE: No

20   (vi) ORIGINAL SOURCE:

       (B) STRAIN:

       (C) INDIVIDUAL ISOLATE:

       (D) DEVELOPMENTAL STAGE:

       (F) TISSUE TYPE:

25   (G) CELL TYPE:

       (H) CELL LINE:

    (ix) FEATURE:

       (A) OTHER INFORMATION: Represents   nucleic   acids

                                           encoding mutant protein of

30   sequence ID No. 12.

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO.13:

   AGCCACCTGA AGTCCAACGA GTGGTTCTCT ACCGCCCGCG ATAAAAAACT   50

   CATGTTCAAG AC   62

   (15) INFORMATION FOR SEQ ID NO:14:

-49-

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 13
            (B) TYPE: amino acid
            (C) STRANDEDNESS:
 5   (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE:
            (A) Description: peptide
      (iii) HYPOTHETICAL: No
       (iv) ANTISENSE: No
10   (v) FRAGMENT TYPE: internal fragment
       (vi) ORIGINAL SOURCE:
            (B) STRAIN:
            (C) INDIVIDUAL ISOLATE:
            (D) DEVELOPMENTAL STAGE:
15   (F) TISSUE TYPE:
            (G) CELL TYPE:
            (H) CELL LINE:
       (ix) FEATURE:
            (A) OTHER INFORMATION: Represents mutant protein of
20   amino acids 381-393 of the
                                      p53 protein.
       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:
            Asn Glu Val Met Trp Lys Thr Lys Trp Pro Asp Ala His
            1   5   10   .

25 (16) INFORMATION FOR SEQ ID NO.15:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 73
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double stranded
30   (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE:
            (A) Description: other
      (iii) HYPOTHETICAL: No
       (iv) ANTISENSE: No
35   (vi) ORIGINAL SOURCE:
            (B) STRAIN:

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(C) INDIVIDUAL ISOLATE:

(D) DEVELOPMENTAL STAGE:

(F) TISSUE TYPE:

(G) CELL TYPE:

5   (H) CELL LINE:

(ix) FEATURE:

(A) OTHER INFORMATION: Represents   nucleic   acids
                          encoding mutant protein of
                          sequence ID No. 14.

10   (xi) SEQUENCE DESCRIPTION: SEQ ID NO.15:

GTCTACCTCC CGCCATAACG AAGTCATGTG GAAGACAAAA TGGCCTGACG   50

CACACTGACA TTCTCCACTT CTT   73

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Claims

1.   An isolated DNA molecule, wherein said molecule
is a synthetic mutated Rb gene encoding a mutated functionally
active retinoblastoma protein.

5   2.   The molecule of claim 1, wherein said gene is
mutated by changing nucleotides that encode for amino acids in
conserved homology regions of the Rb gene.

3.   The molecule of claim 2, wherein said conserved
homology region of the Rb protein is selected from the group
10   consisting of P1, P3 and P5.

4.   The molecule of Claim 1, wherein said gene encodes
an amino acid sequence selected from the group consisting of the
sequences shown in Seq. ID. Nos. 1 and 2.

5.   The molecule of claim 1, wherein said gene is
15   mutated by changing the nucleotide sequence that encodes amino
acids in phosphorylation sites of the Rb protein.

6.   The   molecule   of   claim   5,   wherein   said
phosphorylation sites are selected from the group consisting of
T005, S230, S249, T252, T373, S567, S608, S612, T773, S780,
20   S788, S795, S807, S811, T821 and T826.

7.   An isolated DNA molecule, wherein said molecule
is a synthetic mutated p53 gene encoding a mutated functionally
active p53 protein.

8.   The molecule of claim 7, wherein said gene is
25   mutated by changing the nucleotide sequence that encodes for
amino acids in conserved homology regions of the p53 protein.

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9.   The molecule of claim 8, wherein said conserved homology region of the p53 protein is selected from the group consisting of P1, P3 and P5.

10.   A plasmid comprising the DNA of Claim 1.

5   11.   A plasmid comprising the DNA of Claim 7.

12.   A plasmid adapted for expression in a recombinant cell comprising cDNA encoding for the amino acid sequence of Seq. ID. No. 1 and regulatory elements necessary for expression of the 10   cDNA in the cell.

13.   A plasmid adapted for expression in a recombinant cell comprising cDNA encoding for the amino acid sequence of Seq. ID. No. 2 and regulatory elements necessary for expression of the cDNA in the cell.

15   14.   A method for the treatment of pathological cell proliferative diseases comprising administration to a non-cancerous proliferating cell of the DNA molecule of claim 1.

15.   The method of claim 14, wherein said gene is mutated by changing the nucleotide sequence that encodes amino 20   acids in conserved homology regions of the Rb gene.

16.   The method of claim 15, wherein said conserved homology region of the Rb gene is selected from the group consisting of P1, P3 and P5.

17.   The method of Claim 16, wherein said gene encodes 25   an amino acid sequence selected from the sequences shown in Seq. ID. No. 1 and 2.

18.   The method of claim 14, wherein said gene is mutated by changing the nucleotide sequence that encodes amino acids in phosphorylation sites of the Rb protein.

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19. The method of claim 18, wherein said phosphorylation sites are selected from the group consisting of T005, S230, S249, T252, T373, S567, S608, S612, T773, S780, S788, S795, S807, S811, T821 and T826.

5   20. A method for the treatment of pathological cell proliferative diseases comprising administration to a non-cancerous proliferating cell of the DNA molecule of claim 7.

21. The method of claim 20, wherein said gene is mutated by changing the nucleotide sequence encoding amino acids 10   in conserved homology regions of the p53 protein.

22. The method of claim 21, wherein said conserved homology region of the p53 protein is selected from the group consisting of P1, P3 and P5.

23. The method of claim 20, wherein said non-cancerous 15   proliferating cell disease is selected from the group consisting of psoriasis, benign proliferative skin diseases, ichthyosis, papilloma, basal cell carcinoma, squamous cell carcinoma, fibroproliferative diseases, vasoproliferative diseases and dermatoproliferative diseases.

20·   24. A method of treating malignant cell diseases in individuals comprising administration into a proliferating cancer cell of a DNA molecule of claim 1.

25. The method of claim 24, wherein said gene is mutated by changing the nucleotide sequence encoding amino acids 25   in conserved homology regions of the Rb gene.

26. The method of claim 25, wherein said conserved homology region of the Rb gene is selected from the group consisting of P1, P3 and P5.

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27.   The method of Claim 24, wherein said gene encodes an amino acid sequence selected from the sequences shown in Seq. ID. No. 1 and 2.

28.   The method of claim 24, wherein said gene is 5   mutated by changing the nucleotide sequence encoding amino acids in phosphorylation sites of the Rb protein.

29.   The   method   of   claim   28,   wherein   said phosphorylation sites are selected from the group consisting of T005, S230, S249, T252, T373, S567, S608, S612, T773, S780, S788, 10   S795, S807, S811, T821 and T826.

30.   A method of treating malignant cell diseases in individuals comprising administration into a proliferating cancer cell of a DNA molecule of claim 7.

31.   The method of claim 30, wherein said gene is 15   mutated by changing the nucleotide sequence encoding amino acids in conserved homology regions of the p53 protein.

32.   The method of claim 31, wherein said conserved homology region of the p53 protein is selected from the group consisting of P1, P3 and P5.

20   33.   The method of claim 30, wherein said proliferating cancer cell is a cause of a disease selected from the group consisting of ovarian cancer, bladder cancer, lung cancer, cervical cancer, breast cancer, prostate cancer, glioma, fibrosarcoma, retinoblastoma, melanomas, soft tissue sarcomas, 25   osteosarcoma, colon cancer, carcinoma of the kidney and pancreatic cancer.

34.   A method for the treatment of focal cell proliferative diseases comprising administration of a mutated Rb gene of claim 1 to proliferating cells.

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35.   The method of Claim 34, wherein said proliferating cell is the cause of a disease selected from the group consisting of fibroproliferative, vasoproliferative and neoplastic diseases in the eye.

5   36.   A method for the treatment of focal cell proliferative diseases comprising administration of the mutated p53 gene of claim 7 to proliferating cells.

37.   The method of Claim 36, wherein said proliferating cell is the cause of a disease selected from the group consisting 10   of fibroproliferative, vasoproliferative and neoplastic diseases in the eye.

38.   A mutated retinoblastoma protein produced by the gene of claim 1.

39.   A mutated P53 protein produced by the gene of 15   claim 7.

40.   A method for the treatment of pathological cell proliferative diseases comprising administration concurrently or consecutively to a non-cancerous proliferating cell a mutated Rb gene and a mutated P53 gene.

20   41.   A method of treating malignant cell diseases in individuals comprising administration concurrently or consecutively into a proliferating cancer cell of a mutated Rb gene and a mutated P53 gene.

42.   A method of inducing apoptosis, comprising the 25   step of administering the protein of claim 39 to a cell.

43.   A method of treating a tumor cell having a mutation of a cyclin protein, comprising the step of administering the protein of claim 39 to a cell.

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44.   The method of claim 43, whether said cyclin is selected from the group of cyclin D1, cyclin D2 and cyclin D3.
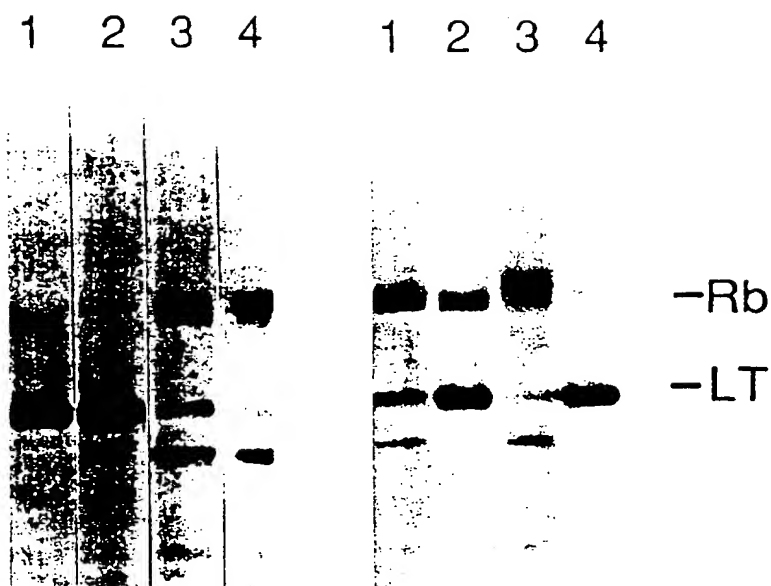
FIG. 1

FIG. 2A
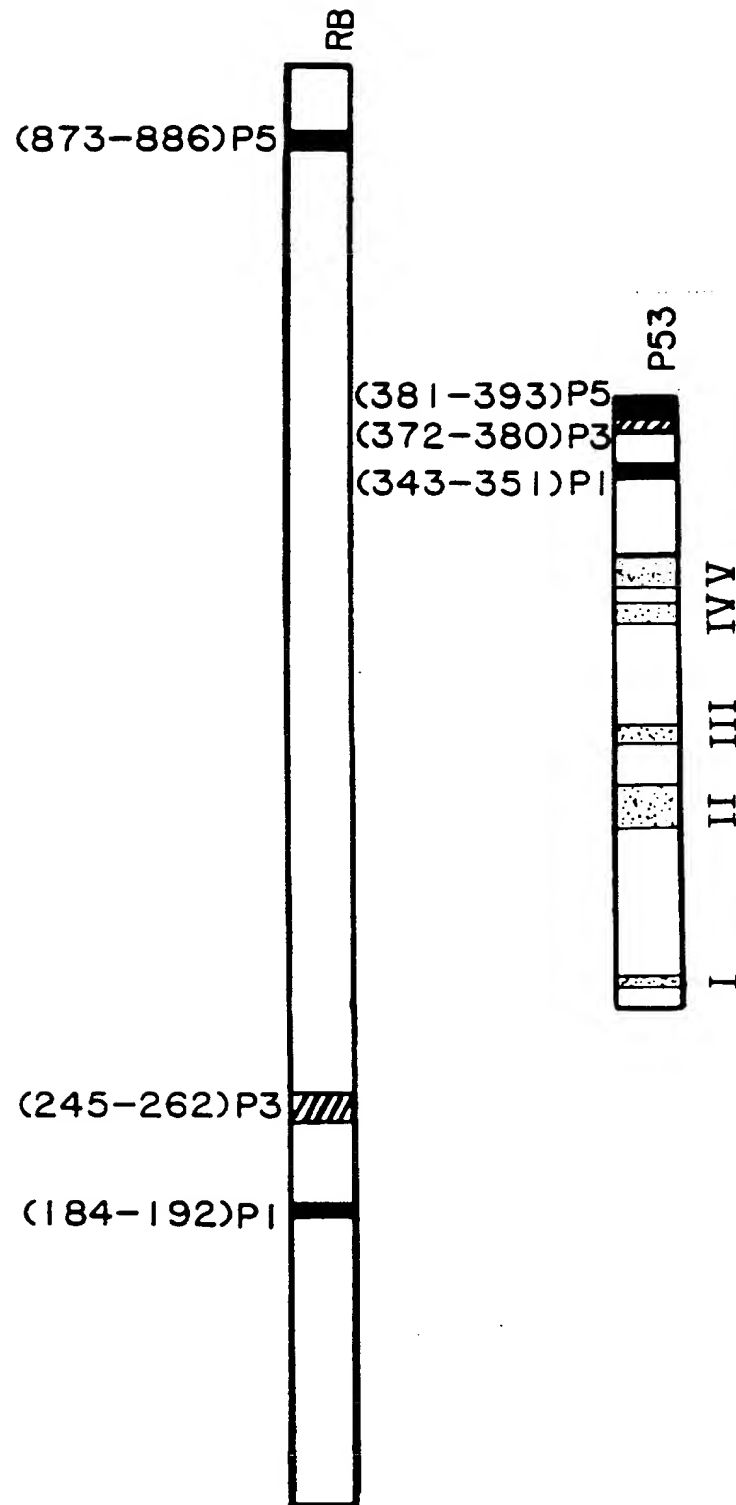
FIG. 2B

1 2 3 4 5 6 7 8 9 10

FIG. 3

1   2   3   4   1   2   3   4

−Rb

−LT

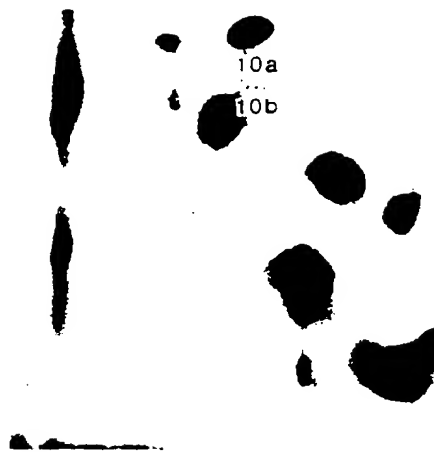# FIG. 5A   FIG. 5B

FIG. 4A

FIG. 4B
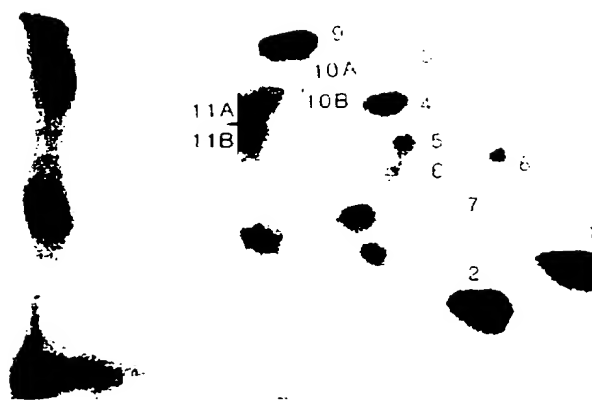

FIG. 4C

FIG. 4D

LT unbound (Rb^E)   LT bound (Rb^H)

FIG. 6

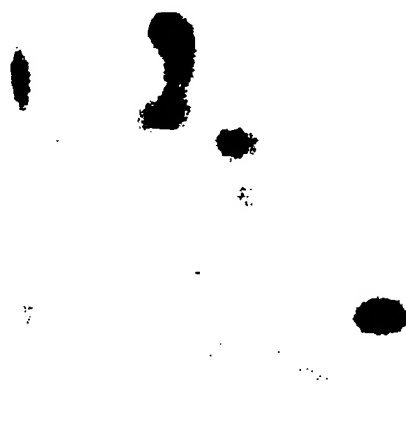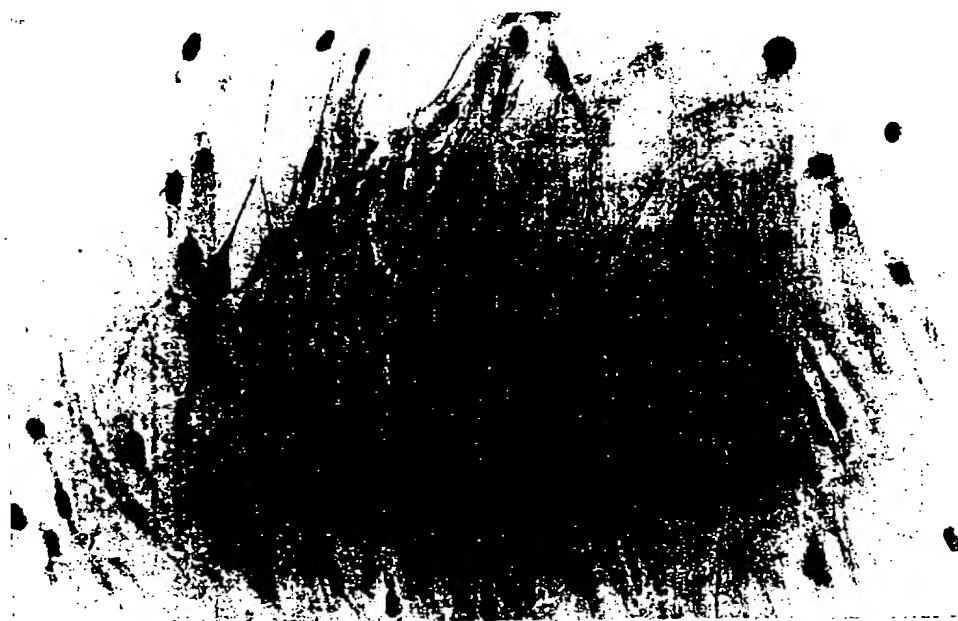Untreated   TGFB treated
                             for 48 hrs

FIG. 7A   FIG. 7B

FIG. 8A

FIG. 8B

FIG. 9A

FIG. 9B

Binding to DNA, target Proteins   D/RNA annealing
Transaction/Growth arrest etc   S₃₁₅ kinasing

Domain   F   Domain   R

Homology
Domain

Wild type (Domain F hidden, Domain R exposed)

S₃₁₅

Mutant (Domain F exposed, Domain R hidden)

FIG. 10

	WT			Mutant(CD7)			
1	2	3	4	5	6	7	8

<-- WT   p53

<-- mutant p53

<-- Cyclin D1,2,3

FIG. 11

WT   3C   3U   5C   5U

FIG. 12A

WT   3C   3U   5C   5U

FIG. 12B

	WT	P5	P3	P4	0	WT	P5	P3	P4
Lysate (ul)	5	5	5	5	5	1	1	1	1

FIG. 13A

	WT		P5		P3		P4	
Ab DO-1(ul)	2	4	2	4	2	4	2	4

FIG. 13B

FIG. 14A

FIG.14B

FIG. 14C

FIG. 14D

FIG. 14E

FIG. 14F

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6)   :C07H 21/04; A61K 31/70, 38/17; C12N 15/85; C07K 14/475
US CL   :514/44, 7, 12; 536/23.5; 530/352; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B.   FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44, 7, 12; 536/23.5; 530/352; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C.   DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,942,123 (LEE et al.) 17 June 1990, column 10, lines 42 to 61, and column 11, lines 23-31.	1-44
Y	Science, Volume 265, issued 15 July 1994, Cho et al., "Crystal Structure of a p53 Tumor Suppressor-DNA Complex: Understanding Tumorigenic Mutations," pages 346-355, see entire document.	7-9, 11, 20-23, 30-33, 36, 37, 3-44
Y	Cancer, Volume 70, issued 1992, Friedmann, "Gene Therapy of Cancer Through Restoration of Tumor-Suppressor Functions?," pages 1810-1817, see pages 1813-1816.	1-41
Y	THE LANCET, Volume 339, issued 21 March 1992, Gutierrez et al., "Gene Therapy for Cancer," pages 715-721, see pages 719-720.	1-41

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 APRIL 1996	23 APR 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	CHARLES C. P. RORIES
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)☆

	International application No.
	PCT/US95/17085

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,049,386 (EPPSTEIN et al.) 17 September 1991, column 10, lines 12-20 and 43-62.	1-44
Y	Molecular and Cellular Biology, Volume 13, No. 6, issued June 1993, Hiebert, "Regions of the Retinoblastoma Gene Product Required for its Interaction with the E2F Transcription Factor Are Necessary for E2 Promoter Repression and pRb-Mediated Growth Suppression," pages 3384-3391, see entire document.	1-6, 10, 12-19, 24-29, 34, 35, 38, 40, 41
Y	Molecular and Cellular Biology, Volume 13, No. 8, issued August 1993, Zacksenhaus et al., "A Bipartite Nuclear Localization Signal in the Retinoblastoma Gene Product and its Importance for Biological Activity," pages 4588-4599, see entire document.	1-6, 10, 12-19, 24-29, 34, 35, 38, 40, 41

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/17085

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)☆

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, BIOSIS, DERWENT WORLD PATENT INDEX, IG SUITE

search terms: retinoblastoma, rb, prb, p53, muta?, residue, amino acid, cdna, protein, administ?, suppress?, tumor, cancer, treat, therapy, 'herapeutic?, cationic(w)lipid, liposome

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-6, 10, 12-19, 24-29, 34, 35, and 38, drawn to a mutant Rb gene and protein, and to methods for using said mutant Rb gene, classified in Class 514, subclass 44, for example.

II. Claims 7-9, 11, 20-23, 30-33, 36, 37, and 39, drawn to a mutant p53 gene and protein, and to methods for using said mutant p53 gene, classified in Class 514, subclass 44, for example.

III. Claims 40 and 41, drawn to a therapeutic method wherein mutant Rb and p53 genes are used together, classified in Class 514, subclass 44, for example.

IV. Claims 42-44, drawn to a therapeutic method wherein mutant p53 protein is administered, classified in Class 514, subclass 12, for example.

The mutant p53 gene and protein, and the methods for using said mutant p53 gene, are included together in Group II because the search of the methods along with the mutant p53 gene and protein is not considered to place an undue burden on the examiner.

The claims of Groups I, II, III, and IV lack unity of invention under 37 C.F.R. 1.475(d), as the claims of Groups II, III, and IV are each drawn to a product that is chemically different from the first-to-be claimed mutant Rb gene product of Group I, and/or are drawn to a method of using said product that is chemically different from the mutant Rb gene product of Group I. Accordingly, the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2, so as to form a single inventive concept.